

Strigolactones perception and signal transduction in the moss Physcomitrium patens

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Strigolactones perception and signal transduction in the moss *Physcomitrium patens*

Thèse de doctorat de l'université Paris-Saclay

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RESUME DES TRAVAUX PRESENTES DANS LE MANUSCRIT DE THESE

Les strigolactones (SL) constituent la neuvième classe d'hormones végétales. Chez les Angiospermes, ces hormones jouent divers rôles dans le développement végétatif et sont des régulateurs majeurs de l'architecture aérienne et racinaire. Leur effet le plus notable est la répression de l'activité des bourgeons axillaires qui se traduit par une moindre ramification des tiges.

Pour autant, les SL n'ont été caractérisées en tant que phytohormones qu'en 2008. En effet, cette famille de molécules avait été auparavant mise en évidence en sa qualité de signal interspécifique, libéré dans la rhizosphère. Les SL jouent ce rôle dans le cadre de deux types d'interactions : le parasitisme inter-végétal et la symbiose mycorhizienne à arbuscules (AMS).

L'implication des SL lors du parasitisme fut le premier mécanisme découvert dans les années 1960, lorsque C.E. Cook et ses collègues isolèrent des molécules jusque-là inconnues à partir d'exsudats racinaires de coton. Ces molécules qui faisaient germer les graines de l'Angiosperme parasite *Striga lutea* furent nommées strigol et strigyl acétate, en l'« honneur du parasite ». Une trentaine d'années plus tard, suite à l'isolement de plusieurs composés proches chez de nombreux hôtes des *Striga*, une équipe japonaise mit en évidence l'action similaire d'une molécule structurellement apparentée, l'orobanchol, exerçant son effet sur la germination des graines de la plante parasite *Orobanche minor* (autre famille que *Striga* parmi les *Orobanchaceae*). Du fait de leur structure proche de celle du strigol, contenant deux groupements lactones, ces molécules furent collectivement baptisées strigolactones.

Ces découvertes soulevèrent une question qui tarauda de nombreux scientifiques jusqu'au milieu des années 2000 et qui peut être résumée ainsi : « Pourquoi diable la sélection naturelle n'a-t-elle pas mis un terme à la production de ces molécules chez les plantes hôtes ? ». C'est donc en 2005, que l'équipe de Kohki Akiyama mit en évidence qu'une autre strigolactone exsudée dans la rhizosphère du lotier, le 5-désoxystrigol, induisait la ramification des hyphes du champignon *Gigaspora margarita*, symbiote de nombreuses plantes dans le cadre de l'AMS. Ce résultat fut ensuite répliqué avec d'autres strigolactones et l'effet positif des SL sur la croissance fongique partiellement élucidé. Ces découvertes indiquent que l'effet positif des SL sur la symbiose l'emporte sur leur effet négatif dans le cadre du parasitisme par les *Orobanchaceae* et expliquent la conservation des SL au cours de l'évolution des plantes terrestres.

Etant donné le rôle majeur des SL dans l'induction de l'AMS, une symbiose à l'origine ancienne et quasiomniprésente parmi les plantes terrestres, il n'est pas étonnant de retrouver la capacité de biosynthèse de SL au sein de tous les taxons. En effet, les gènes codant les premières enzymes de biosynthèse des SL (D27, CCD7 et CCD8) sont retrouvés chez les Bryophytes (mousses, hépatiques et anthocérotes), les Lycophytes (sélaginelles, lycopodiales et les isoëtes), les Monilophytes (fougères et prêles) et les Spermatophytes (Gymnospermes et Angiospermes), et même chez certaines algues Spermatophytes (algues les plus proches des plantes terrestres). Il est donc tentant de penser que la fonction ancestrale des SL était de promouvoir la symbiose chez les premières plantes terrestres. De plus, cette hypothèse semble être confirmée par l'absence d'homologues canoniques des gènes codant le récepteur des SL (D14) et les acteurs régulant ensuite la transduction du signal (SMXL7) en dehors des Spermatophytes (voire des Angiospermes). Seule la protéine à boîte F MAX2, agissant comme corécepteur des SL, est retrouvée chez toutes les plantes terrestres. L'absence du récepteur D14 et du répresseur SMXL7 indiquerait que les SL n'ont pas de fonction phytohormonale chez les plantes terrestres souvent présentées comme « basales » (non-Spermatophytes, qui ne produisent pas de graines). Au contraire, toutes les plantes terrestres possèdent, en plus de MAX2, un homologue plus lointain de D14 appelé KAI2. Chez les Angiospermes, KAI2 et MAX2 agissent ensemble avec SMAX1 (un autre homologue SMXL) dans la voie de réponse à un autre signal, le KAI2-ligand (KL). Le KL est une phytohormone encore non-identifiée qui peut être mimée par les karrikines (KAR), des molécules produites à partir de la combustion de matériel végétal lors des feux de forêt. Les KAR induisent la germination des graines chez certaines espèces, notamment chez la plante modèle Arabidopsis. Hormis ce rôle de promotion de la germination, KAR et KL régulent le développement précoce des plantules et il a récemment été montré que la voie KAI2-MAX2-SMAX1 est nécessaire à l'instauration de l'AMS chez les Angiospermes. Les études phylogénétiques récentes semblent indiquer que cette voie KL est ancestrale. Cependant, le rôle de cette voie en dehors des plantes à graines reste à déterminer, ainsi que celui qu'elle jouait chez les plantes terrestres ancestrales. Ces éléments d'introduction sont développés dans le chapitre III.

A ce jour, seule une espèce de Bryophytes a fait preuve d'une réponse développementale à un traitement avec des SL : la mousse *Physcomitrium patens* (*P. patens*). Le développement de *P. patens* et son utilisation en tant que plante modèle en biologie sont décrits dans le chapitre I. Les rôles et voies cellulaires associées aux phytohormones chez *P. patens* sont détaillés dans le chapitre II. En outre, il est important de souligner que les mousses ne recourent pas à la symbiose avec des champignons endomycorhiziens. Donc, la réponse développementale observée chez cette espèce n'est pas le fait d'un défaut d'association symbiotique. *P. patens* réagit notamment à la présence de faibles quantités de SL par une diminution de la ramification et de la croissance de son *protonema* (phase développementale filamentaire caractéristique des mousses). Lorsque le gène de biosynthèse des SL *CCD8* est muté chez *P. patens*, la plante développe un phénotype de déficience en SL, révélé notamment par une croissance et une ramification exacerbée du *protonema*.

Comme toutes les Bryophytes, *P. patens* ne possède pas d'homologue proche de D14 et de SMXL7, mais possède un homologue de MAX2 (PpMAX2), ainsi que de nombreux homologues de KAI2 (PpKAI2Like ou PpKAI2L-A à -M) et quatre homologues SMAX1/SMXL7 (PpSMXLA à D). L'expansion des familles KAI2L et SMXL est une spécificité des mousses parmi les plantes non-Spermatophytes. Etant donné que *P. patens* est capable de répondre aux SL, une hypothèse possible est que PpMAX2, certaines copies PpKAI2L et certaines copies PpSMXL seraient impliquées dans la voie de signalisation des SL à la manière de ce qui est connu chez les Angiospermes. Cependant, le mutant perte-de-fonction *Ppmax2* ne présente pas un phénotype développemental similaire à *Ppccd8*, ce qui est pourtant attendu pour un mutant de réponse aux SL. Au contraire, *Ppmax2* a un *protonema* peu développé et passe rapidement à la phase développementale suivante, c'est-à-dire la formation de tiges feuillées (gamétophores) qui vont porter les organes reproducteurs. En outre, *Ppmax2* peut toujours répondre à un ajout de SL exogènes, ce qui démontre clairement que la protéine PpMAX2 n'est pas nécessaire pour cette réponse.

Une caractérisation fine de ce mutant suggère que PpMAX2 est impliquée dans la réponse à la lumière et probablement dans la voie de signalisation du KL, ce qui est également appuyé par les travaux de thèse ici présentés (chapitres IV, VI et VII). Une étude préliminaire portant sur les protéines PpKAI2L suggère pourtant que si certaines sont largement similaires à KAI2 et donc potentiellement impliquées dans la perception du KL, d'autres peuvent être des récepteurs de SL car leur structure les rapproche de D14. Les prédictions de cette étude sont ici largement confirmées dans le chapitre IV. *P. patens* possède quatre clades de PpKAI2L : le clade (A-E) est impliqué dans la voie PpMAX2-dépendante, le clade (G, J, M) est nécessaire à la perception des SL, alors que les clades (F, K) et (H, I, L) exercent des fonctions restant à élucider. Ces conclusions reposent sur l'étude de mutants multiples *Ppkai2-L* et de leur capacité à

répondre à une SL artificielle ou à un mime de KL artificiel, ainsi que sur la caractérisation biochimique de certaines protéines PpKAI2L.

Dans le chapitre VI, le rôle des protéines PpSMXL dans la voie de réponse aux SL et dans la voie PpMAX2dépendante est exploré, par la caractérisation du développement de mutants *Ppsmxl* et de lignées sur-exprimant les gènes *PpSMXL*, ainsi que par des approches de recherche d'interactions protéine/protéine. Les protéines PpSMXL sont séparées en deux clades (A/B et C/D) mais semblent toutefois avoir la même fonction d'inhibiteurs de la voie PpMAX2dépendante. Cependant, il apparaît que la voie de signalisation des SL agit également via PpSMXLC/D car les doubles mutants *Ppsmxlcd* ne répondent plus à un traitement avec des SL artificielles. Dans le chapitre VII, le rôle des protéines PpSMXL en situations de stress (blessure et exposition au froid) est investigué et achève de mettre en évidence un rôle primordial et particulier de la protéine PpSMXLC dans l'équilibre entre croissance et tolérance au stress. Les rôles hypothétiquement opposés des SL et du KL sur la division cellulaire sont également explorés.

En conclusion, il apparaît que la mousse *P. patens* n'utilise pas la même voie de signalisation des SL que les Angiospermes. L'expansion de la famille des KAI2L au fil des duplications de génomes dans la lignée évolutive des mousses a permis l'émergence d'une nouvelle fonction : la perception des SL. Il est intéressant de noter que l'émergence de la germination SL-dépendante chez les plantes Angiospermes parasites est expliquée par un mécanisme analogue. Les copies additionnelles de SMXL chez les mousses, ainsi que l'unique copie de MAX2, n'ont pas été recrutées pour transduire le signal SL au cours de l'évolution des mousses, à l'inverse de ce qui est probablement advenu au cours de l'évolution des Angiospermes. Par ailleurs, l'existence d'une voie similaire à la voie KAI2-MAX2-SMAX1 des Angiospermes chez la mousse *P. patens* appuie l'hypothèse selon laquelle cette voie KL est ancestrale chez les plantes terrestres. En outre, la voie KL semble jouer un rôle majeur dans le développement de *P. patens*, ce qui pourrait indiquer qu'elle jouait également un rôle très important chez les premières plantes terrestres, probablement plus semblables aux Bryophytes qu'aux Angiospermes dans leur développement.

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OVERVIEW OF THE MANUSCRIPT CONTENTS

The introduction starts with a rapid presentation of the model organism that was used along this thesis work: the moss *Physcomitrium patens* (Chapter I). Then, phytohormones biosynthesis and signaling pathways, as well as phytohormones roles in this model Bryophyte's development are explored, in a thorough literature review presented in chapter II (following chapters can be read independently from chapter II). Finally, we focus on strigolactones in chapter III, first by a quick reminder about how these molecules were identified and about their known roles in plants. This chapter ends with considerations about the evolution of strigolactones biosynthesis and signaling in land plants and about the multiple connections between strigolactones and the mysterious KAI2-ligand phytohormone.

In chapter IV are presented our findings on putative receptors of strigolactones and KAI2-ligand in *Physcomitrium patens*, which main conclusion is that different functional subclades have emerged from the same family of proteins and eventually enabled response to strigolactones and to KAI2-ligand in this moss evolutive history. I actively participated in the generation of results, reflection and redaction resulting in this chapter. Chapter V focuses on current knowledge about SMXL proteins, a common player found in strigolactones and KAI2-ligand signaling pathways. Hypotheses about these proteins molecular function, in the context of strigolactones/KAI2-ligand signaling, are raised. Chapter VI presents the main results obtained through the study of *Physcomitrium patens SMXL* genes, which was the main focus of this thesis project. We report that SMXL homologs of this moss do not act as repressors of SL response, but rather as repressors of response to KAI2-ligand. However, they do play a positive role in response to strigolactones, which is an unexpected finding that will be further investigated. In chapter VII, the role of these SMXL homologs in growth restriction is further characterized in the context of wound-induced regeneration or of moderate cold stress. The putative roles of strigolactones and KAI2-ligand in this process are also investigated.

In chapter VIII, findings from chapters IV-VII are replaced in an evolutive scope and the function of strigolactones in mosses and in ancestral land plants is discussed.

INTRODUCTION

Chapter I - Physcomitrium patens life cycle and usefulness in research

I-A) Physcomitrium patens phylogenetic location

Bryophytes are non-vascular descendants of the earliest diverging land plants. Land plants emergence is dated around 500 Mya, while the vascular plants (tracheophytes) lineage diverged approximately 450 Mya (Morris et al., 2018). Phylogenetic relationships between the three classes of bryophytes, namely mosses (*Bryopsida*), liverworts (*Marchantiopsida*) and hornworts (*Anthocerotopsida*), have long been a subject of debate. Nevertheless, bryophytes now tend to be considered as a monophyletic clade, named *Bryophyta* (Puttick et al., 2018; de Sousa et al., 2019, 2020), relative to vascular plants (see figure I-1).



Figure I-1 – Phylogenetic relationships in the green lineage (*Viridiplantae*)

I-B) Physcomitrium patens as a model organism in biology

Together with the liverwort Marchantia polymorpha, the moss Physcomitrium (or Physcomitrella) patens has since long outreached from the field of bryology into diverse plant biology studies as a major model species (Rensing et al., 2020). This phenomenon is explained by the relatively simple life cycle and development of this moss, while still showing numerous similarities to other land plants, particularly at the cellular level (Schaefer and Zrÿd, 2001; Wood et al., 2000). In addition, P. patens has a particularly high regeneration ability, making in vitro propagation quick and convenient (Cove, 2005). Thanks to the sequencing of its genome (Rensing et al., 2008), as well as to its high frequency of homologous recombination (Schaefer and Zrÿd, 1997; Schaefer, 2001), P. patens is particularly amenable to genome modifications and thus to reverse genetics. While *P. patens* has been shown to be transformable by diverse methods including Agrobacterium mediated transfection (Cove et al., 2009b; Li et al., 2010) and biolistic based methods (Smidkova et al., 2010), its robust protoplasts (Cove, 2005) are most often transformed through poly-ethylene-glycol (PEG)-mediated membrane permeabilization (Hohe and Reski, 2002). Moreover, modification of several loci at once has been achieved very efficiently via the use of CRISPR-Cas based systems in P. patens, that is seemingly becoming more prevalent over homologous recombination (Lopez-Obando et al., 2016b; Collonnier et al., 2017; Mallett et al., 2019; Yi and Goshima, 2019; Pu et al., 2019; Veillet et al., 2020). Forward genetics methods have also been proven to work well in this species, as collections of randomly mutagenized P. patens mutants exist, obtained using transposons (Egener et al., 2002; Vives et al., 2016; Mohanasundaram et al., 2019) or T-DNA (Cove et al., 2009b). Several reports also showed that *P. patens* is amenable to chemical (alkylating agents such as EMS), genotoxins (Holá et al., 2013), and radiation (X-rays and UV) triggered mutagenesis (Engel, 1968; Cove et al., 2009a). Most importantly, the genome of P. patens is in a haploid state for the longest part of the life cycle. In other words, the gametophytic stage is dominant over the sporophytic stage in bryophytes, contrarily to vascular plants. That makes mutant generation even more convenient since possible mutant phenotypes are visible immediately (at least when the mutant is regenerated from a single mutagenized cell, e.g. a protoplast) without there being a need for a round of self-fertilization to generate homozygotes. This is especially valuable since the fertility of the laboratory multiplied Gransden ecotype has significantly decreased along years (Rensing et al., 2020). However, this advantage can become a hindrance when vital genes are targeted. Fortunately, knock-down alternatives are a part of the well-tried toolkit in *P. patens* (Khraiwesh et al., 2008; Nakaoka et al., 2012) and can circumvent this issue, as well as the generation of conditional mutants using inducible promoters (Kubo et al., 2013). Additionally, somatic diploids can be induced to resolve this issue (Rensing et al., 2020).



Figure I-2 – Physcomitrium patens cycle of life

I-C) Physcomitrium patens cycle of life

P. patens gametophytic life stage starts from the germination of a spore under appropriate environmental conditions, the imbibition level and amount of red light being crucial (Cove et al., 1978). The first tissue that is generated from the germinating spore is primary *chloronema*, composed of cells with a high chloroplast content and disposed in a single cell file (filaments) (figure I-2). This filament elongates *via* division and longitudinal elongation of the apical cell (tip-growth) and branches *via* division of subapical cells. Apical cells of these first produced filaments eventually switch to producing another type of tip-growing filament, which is faster growing and has a decreased chloroplast content: the *caulonema* branches similarly to *chloronema* and gives rise mainly to secondary *chloronema*. Aside from the aforementioned differences between the two types of filaments, another divergence is the orientation of cross walls: *chloronema's* are perpendicular to the elongation axis, while *caulonema* make up the *protonema*, which can be seen as *P. patens* first stage of gametophytic life, enabling superficial colonization of the medium (Cove, 2005). Environmental cues that are not clearly understood, together with better known endogenous signals (notably cytokinins (Reski and Abel, 1985), see the following literature review on phytohormones in mosses in chapter II), can trigger the differentiation of branch cells initials emerging from *caulonema* into bud progenitor cells. These cells acquire the ability to divide asymmetrically and eventually lead to the formation of leafy shoots called gametophores, switching *P. patens*

development pattern to three-dimensional growth. Gametophores develop initial cells of phyllids (proto leaves) by successive divisions of their apical meristematic cell (Harrison et al., 2009). Phyllids develop following a spiral phyllotaxis on the gametophore stem and are made of two main parts: the lamina, which is a monolayer of highly photosynthetic cells, and the central multilayered midrib which contains water-conducting tissues (hydroids) (Crandall-Stotler and Bartholomew-Began, 2007). The gametophore stem epidermis, at the axil of phyllids in the continuity of the midrib (Sakakibara et al., 2003), generates another type of filaments: the anchoring rhizoids. In P. patens, rhizoids are unbranched, reddish-brown pigmented filaments, and structurally very similar to *caulonema*, also having oblique cross walls (Sakakibara et al., 2003). Also at the axil of phyllids, but on the adaxial side, axillary hairs develop from the epidermis (Eklund et al., 2010). However, the function of these structure is still under investigation. The stem epidermis can moreover occasionally give rise to adventitious gametophores (or branches) (Eklund et al., 2010; Coudert et al., 2015). Ultimately, each gametophore differentiates several male (antheridia) around female (archegonia) reproductive organs at its apex, when environmental conditions become suitable (low temperature and short daylength (Hohe et al., 2002; Landberg et al., 2013)). After fertilization of the oosphere in the archegonia (usually self-fertilization) a diploid zygote is formed, most often from a single archegonia per apex (Hohe et al., 2002). The success rate of fertilization is highly reliant on a high hygrometry level so that the sperm cells can access the egg cell in the archegonia (Kofuji et al., 2018; Hiss et al., 2017). The zygote undergoes several rounds of mitosis, giving rise to the diploid sporophytic stage of *P. patens* life. However, the sporophyte never lives independently from its maternal gametophyte and keeps getting nutrients from it through their interconnecting seta. This stage is very brief as specific cells of the sporangium soon undergo meiosis and produce haploid spores. When the sporophyte becomes mature and dry, it ruptures, freeing the spores in the environment and starting a new gametophytic cycle. The whole cycle usually takes around 4 months to be completed in the so far most used Gransden ecotype (Engel, 1968).

I-D) Physcomitrium patens usefulness in evo-devo studies

Owing to its early divergence from other land plants as a bryophyte, *P. patens* is widely employed as a model plant species to explore research topics relative to plant evolution. As a matter of facts, despite its ancient divergence with vascular plants, *P. patens* shares many characteristics with them, making it particularly amenable to evo-devo (Evolutionary Developmental Biology) approaches. Studying *P. patens* can hence give precious insight into both the ancestral state and the possible evolutionary fates of diverse conserved processes and structures. Indeed, while organs are notably different between this moss and vascular plants, many parallels can be traced between them. For instance, rhizoids can be associated to vascular plants' root hair, phyllids to leaves photosynthetic parenchyma, and the basic mechanisms underlying meristem maintenance and function can be compared. Moreover, *P. patens* is widely comparable to vascular plants model species at the cellular level, being equally sensitive and displaying similar responses to most growth-regulating compounds (e.g. phytohormones, see the following review in chapter II) and environmental stimuli (light, gravitropism, nutrient deficiency, dehydration, etc.). It is also important to note that even points of divergence between bryophytes and vascular plants can be addressed by using *P. patens* as a model, notably the switch between gametophytic and sporophytic dominance (Pires and Dolan, 2012; Bowman et al., 2016).

There is however a cautionary statement to remember: *P. patens* is not more representative of the ancestral land plants than any other extant land plant (Puttick et al., 2018). Each plant species is the result of a mix of conserved primitive features and derived characters distinctive of its lineage. Only a joint effort, ideally with comparative studies including plants from diverse lineages (from bryophytes and tracheophytes), can give a reliable view of ancestral processes.

Chapter II - Phytohormones biosynthesis and signaling pathways of mosses

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It can be read independently from chapter I and all following chapters without preventing understanding.

Authors contributions:

Both A.G and S.B contributed to compiling and reading appropriate literature and to the writing of the present review paper.

INTRODUCTION

Hormones make up a class of signaling molecules, specific to multicellular organisms. They enable communication between somewhat distant cells of the same organism and thus coordination of distant cells/tissues to trigger given physiological and/or developmental processes. Classically, hormones have been defined in animals as signaling molecules produced by particular tissues or even organs (glands) that act on other cells at a distance, by moving through the blood flow, are perceived at low concentration by specific receptors and induce diverse physiological responses in the target cells (historical definition of Sterling in 1905, also establishing the term hormone). These effects are attained via the activation of an intracellular transduction pathway, and notably act via modulation of gene transcription. The main divergence between animal hormones and plant hormones (also called phytohormones, which concept even predates that of animal hormones as it can be traced back to the 18th century) is that all plant cells are potentially able to synthesize and excrete phytohormones, while only highly specialized cells are dedicated to this function in animals, for each given hormone. Phytohormones can be transported through the vasculature (xylem or phloem) in Tracheophytes, and even by cell-to-cell transport (see Park et al., 2017 for a review on this subject). Perception usually occurs at the plasma membrane or in the cell, mostly depending on the hydrophilic/hydrophobic nature of the signal. Generally speaking, hydrophobic signals act directly in the target cell nucleus and thus do not require the generation of a secondary messenger. Chemical identity of molecules acting as hormones can be very variable, in both animals and plants.

Phytohormones have first been discovered and studied in Angiosperms, notably auxin which presence was evidenced in the 1920s (Went, 1926). Soon, they were found to also be produced in early-diverging, non-vascular plants, which raised new interrogations about their involvement in plant evolution. Pioneer work of Bopp *et al.* starting for the 1950s on *Funaria hygrometrica* (*F. hygrometrica*), and complementary work by Ashton *et al.* and Wang *et al.* and then by Reski *et al.* on *Physcomitrium (Physcomitrella) patens (P. patens)*, starting from the end of the 1970s, paved the way to understanding phytohormones' biosynthesis and action in mosses. The specific ease of *P. patens*' use in genetic studies gradually made this species the favorite model for mosses and even one main model in plant biology (Engel, 1968; Ashton and Cove, 1977). The sequencing of *P. patens* genome (Rensing et al., 2008) added to the potential of this species by broadening its use to reverse genetics studies. In addition to this, most of the decades-old knowledge gained from early studies focused on *Funaria hygrometrica* and *Ceratodon purpureus* has now been transferred to *P. patens*. Hence, we chose to focus on this model species and findings emerging from other mosses will not be reported here unless the information is especially meaningful and/or missing in *P. patens*.

Phytohormones classically refer to nine groups of compounds primarily identified in Angiosperms: auxins (AUX), cytokinins (CK), gibberellins (GA), abscisic acid (ABA), ethylene (ETH), brassinosteroids (BR), salicylic acid (SA), jasmonic acid (JA) and strigolactones (SL). A comprehensive study of phytohormone content on a broad spectrum of 30 Bryophytes species (among which 24 mosses, Drábková et al., 2015) highlighted the presence of AUX, CK, ABA, SA and JA. GA and BR were identified in low quantities and their occurrence in mosses was deemed dubious. SL and ETH were not addressed in this study. Since most of these groups are present in mosses, all nine classical phytohormones groups will be discussed in the present review, as well as CLE signaling peptides, and a possible tenth class of phytohormones that is so far referred to as KAI2-ligand (KL). Other compounds that are sometimes referred to as plant hormones, such as polyamines, strictly intracellular lipidic signals, signaling peptides aside from CLE, some signaling messenger RNAs, cyclic monophosphate nucleotides and small molecules such as Ca²⁺, nitric oxide (NO) and hydrogen peroxide will not be explored here. The reason for ignoring these is either that these molecules are most often considered as second messengers instead of hormones proper, or that evidence of their occurrence and/or function is fragmentary in mosses. Likewise, the signaling function of sugar and nitrate will not be explored here, as they are above all vital compounds.



Figure II-1 - Representative molecules of each phytohormonal group. The structure of the molecule that is the most widely distributed in land plants is given, for each phytohormone family. Molecules (or analogs) from the same family are noted in the same colour: auxins in blue, cytokinins in red, jasmonates in yellow, strigolactones in purple. IAA= Indole-3-Acetic Acid; NAA= 1-Naphthaleneacetic acid ; BA= benzyl adenine; N⁶-iP= N⁶-(Δ^2 -isopentenyl)-adenine; SA= Salicylic Acid; ABA= Abscisic acid; BR= Brassinosteroid ;JA= Jasmonic Acid; OPDA= 12-oxo-phytodienoic acid; CL= Carlactone; KAR1= Karrikin 1 = Karrikinolide.

AUXINS

The predominant and most potent natural auxin is indole-3-acetic acid, shortened as IAA (Figure II-1). Auxin has been identified in evolutionarily diverse species, from filamentous brown algae to flowering plants, and is a wellknown regulator of cell growth and division (Le Bail et al., 2010). While five different pathways for auxin biosynthesis have been proposed in flowering plants, only two appear to exist in P. patens. In 2010, Eklund and colleagues showed that genes involved in auxin biosynthesis from tryptophan through the indole-3-pyruvic acid pathway (TRYPTOPHAN AMINOTRANSFERASE 1 (TAA1)-likes) and from the tryptamine pathway (YUCCAs) have homologs in P. patens genome. It has not yet been formally proven that these genes are involved in auxin production, but some indirect evidence points to the tryptamine pathway being active. Indeed, PpSHI/STY proteins, which homologs in Angiosperms are inducers of YUCCA expression, do have a positive role on auxin biosynthesis in P. patens (Eklund et al., 2010). The major auxin produced in many mosses is IAA (Drábková et al., 2015), but it has not been confirmed in *P. patens* itself. Auxin biosynthesis likely takes place all along *protonema* and its production in gametophores can be ensured by axillary hairs (Eklund et al., 2010). A balance between active and inactive auxin can be achieved through conjugation to amino acids via GRETCHEN HAGEN3 (GH3) enzymes, which number four in P. patens (Ludwig-Müller et al., 2009). The two PpGH3 genes studied are both widely expressed starting early in development but are not induced by auxin application. Nevertheless, PpGH3 genes seem to play a role in auxin homeostasis, as Ppgh3 loss of function mutants are hypersensitive to auxin and accumulate free auxin (Ludwig-Müller et al., 2009).

The first demonstrated effect of auxin on moss development was induction of the *chloronema* to *caulonema* transition (Johri and Desai, 1973; Ashton et al., 1979b) (Figure II-2). In 2006, Decker *et al.* further demonstrated that *caulonema* promotion by auxin is associated to an arrest of the cell cycle in the G1 phase, a specificity of *caulonema*. Moreover, Thelander and colleagues recently showed that auxin inhibits *protonema* branching (Thelander et al., 2018). Aside from this, auxin has many other developmental roles in mosses, such as the promotion of rhizoid formation (Ashton et al., 1979b; Sakakibara et al., 2003). Exogenously supplied 1-Naphthaleneacetic acid (NAA, a synthetic artificial auxin analog) not only increases rhizoid number, but also induces apical rhizoids (Sakakibara et al., 2003). Besides, expression of the HD-ZIP class I transcription factor gene *PpHB7* is rapidly induced by NAA. HD-ZIP transcription factors are largely known as potent regulators of cell differentiation in Angiosperms. PpHB7 is partly responsible for the effect of auxin on rhizoid differentiation, notably by down-regulating total chloroplast mass per cell (Sakakibara et al., 2003). Rhizoid determination, on the other hand, is likely PpHB7-independent. Yet another role of auxin is the regulation of gametophore morphogenesis: at low concentrations, NAA promotes elongation of gametophores stems (Fujita et al., 2008; Bennett et al., 2014) and cell elongation in phyllids (Decker et al., 2006), but both tissues display a different level of sensitivity (Bennett et al., 2014).

Polar auxin transport (PAT) in Angiosperms relies on PIN (PIN-FORMED) efflux transporters, and four *PIN* genes (*PpPINA* to *PpPIND*) have been identified in *P. patens* (Bennett et al., 2014). *PpPIN* genes are expressed in *protonema* with a maximum level in apical cells (Viaene et al., 2014), and the PpPIN proteins themselves are located both at the basal and at the apical membrane in these cells, suggesting auxin is exported acropetally but also exported out of the tip in the medium. In *protonema*, *PpPINA-C* limit intracellular auxin content, thereby delaying the onset of *caulonema* differentiation (Viaene et al., 2014). Bennett *et al.* have demonstrated that *PpPINA* and *PpPINB* function is also needed for proper gametophore development. Interestingly, the gametophores of the *Pppina/b* double mutant are

agravitropic and aphototropic (to blue light) but not its filaments, suggesting PpPIN function is most important in gametophores (Bennett et al., 2014). In gametophores, auxin produced by the main stem exerts an inhibitory effect on branches emergence (Von Maltzahn, 1959; Nyman and Cutter, 1981; Sakakibara et al., 2003). However, auxin production in the apex and PpPIN-mediated exportation cannot explain alone the branching pattern of gametophores. Indeed, the *Pppina/b* mutant is only minorly affected in gametophore branching and PIN inhibitors have no impact on this phenotype (Coudert et al., 2015). Computer modelling proved that only a bidirectional transport of auxin in the stem can generate a realistic branching pattern. Further investigation by Coudert et al. (2015) identified regulation of plasmodesmal conductivity through callose deposition as a mean for bidirectional auxin transport. As for the auxininduced emergence of rhizoids, Thelander and colleagues suggest that a combination of PpPIN-mediated auxin export from the leaves (Sakakibara et al., 2003), and bidirectional plasmodesmal transport in the stem, might allow sufficient auxin accumulation at the base of phyllids to promote rhizoid initiation (Thelander et al., 2018). Auxin production and export have also been shown to be important for apical opening of both archegonia and antheridia, and beforehand during the development of the egg cell (Landberg et al., 2013). Auxin response seems even more tightly spatially and temporally regulated across embryo development which indicates a complex role of auxin during zygote maturation. Finally, *PpPINA* and *PpPINB* are required for proper sporophyte development and notably prevent sporophyte branching (Bennett et al., 2014).

Many genes homologous to auxin signaling components in flowering plants are found in *P. patens* genome (Rensing et al., 2008) (Figure II-3). In vascular plants, auxin is perceived by receptors of the TIR1/AFB family. Then the auxin signal is transduced via the degradation of Aux/IAA repressors, which frees transcription factors of the ARF family, regulating the expression of auxin-responsive genes, P. patens possesses four TIR1/AFB genes, two more distant homologs (PpXFB1 and PpXBF2), three Aux/IAA genes (PpIAA1A, PpIAA1B and PpIAA2, Prigge et al., 2010) and a dozen of ARF genes (Paponov et al., 2009; Lavy et al., 2016; Kato et al., 2017). From the study of transient knock-down RNAi lines, it appears that if PpXFBs are not required for response to auxin, each PpAFB is necessary for response to NAA and therefore a good auxin receptor candidate (Prigge et al., 2010). Despite being larger proteins than flowering plants Aux/IAAs, PpIAA contain all known functional domains, including a consensus EAR motif required for transcriptional regulation and a degron motif necessary for proteasomal degradation (Prigge et al., 2010). Prigge and colleagues examined 17 NAR mutants (NAA-resistant mutants (Ashton et al., 1979a)) and found that the more severe ones were mutated in the DII degron motif of PpIAAs (VGWPPV). In Angiosperms, similar mutations prevent association with the SCF^{TIR1/AFB} ubiquitylation complex, therefore making the Aux/IAA more stable and preventing auxin signal transduction. Thus, PpIAAs do act as negative regulators of auxin response. Moreover, NAA treatment induces expression of all three *PpIAA* genes within one hour (Prigge et al., 2010), revealing that auxin signaling is subjected to negative feedback regulation via *PpIAAs*, which was later evidenced as PpARF-dependent (Tao and Estelle, 2018). Furthermore, the degron motif is necessary for auxin triggered PpAFB/PpIAA interaction (Prigge et al., 2010). Degron deletion in PpIAA1A results in resistance to exogenous NAA but interestingly does not cause a mutant phenotype in the absence of treatment, e.g. in response to basal levels of endogenous IAA (Tao and Estelle, 2018). Transduction of the auxin signal also requires TPL/TPRs, one of the two major classes of plant transcriptional corepressors, in P. patens: PpTPL1 and PpTPL2 proteins both interact with all 3 PpIAAs, in an EAR motif-dependent fashion (Causier et al., 2012b). However, P. patens lines expressing an EAR-deleted version of PpIAA1A are not as impacted as the triple *ppiaa* mutant, suggesting that EAR is not necessary for all PpIAAs functions, including some

aspects of PpIAA-mediated transcriptional repression (Tao and Estelle, 2018). PB1 domains predicted to enable proteinprotein interactions are found both in PpARFs and PpIAAs. Constitution of PpIAA oligomers and PpIAA-PpARF interactions contribute to repressive function of PpIAA1A but PpIAA1A monomers can also have a repressive activity on transcription (Tao and Estelle, 2018). According to a recent study by Lavy et al., P. patens possesses 16 ARF genes, divided in 4 subgroups, with one being non-seed plant specific (Lavy et al., 2016). As in Angiosperms, there are PpARFs that have a repressive effect on auxin-induced transcriptional response (negative ARFs) and PpARFs that have a positive role (positive ARFs). Both functional types target the same promoter elements and therefore the same genes. Additionally, they both act in coordination with PpIAAs. Moreover, the effect of PpARFs on transcription also relies on TPLs: PpTPL1 and/or PpTPL2 interact in an EAR-dependent manner with negative PpARFs (PpARFe and PpARFf). Another negative PpARF (PpARFb4) does not need the EAR motif to interact with PpTPLs, suggesting that it acts by competitive DNA binding instead of direct repression (Lavy et al., 2016). The repressive effect of negative PpARFs is less stable than that of PpIAAs, which partly explains how PpARFs enable fine-tuning of the auxin response. On the other hand, PpIAAs ensure a long-term repression of transcriptional response to auxin (Lavy et al., 2016). Indeed, Lavy and colleagues showed that IAA treatment has a huge effect on P. patens transcriptome, as more than 700 genes are induced and 700 more are repressed. This is also confirmed by the observation that in the triple *Ppiaa* mutant, which displays a constitutive auxin response and is completely insensitive to auxin, as much as one third of all *P. patens* genes are differentially expressed relative to WT. PpIAA1A is likely the main responsible for this huge effect, since the Ppiaalb Ppiaa2 double mutant is phenotypically indistinguishable from WT (Lavy et al., 2016). Two mechanisms permitting PpIAA-mediated auxin-induced transcriptional repression probably coexist: the first via association with PpARF that could interact with chromatin remodelers, the second via EAR-mediated interaction with PpTPLs that could sustain repression by recruiting histone deacetylases (Tao and Estelle, 2018). Paponov et al. also reported the existence of a PpARF lacking the C-terminal dimerization domain, thus unable to interact with PpIAA (Paponov et al., 2009). This specific PpARF could therefore regulate the basal, auxin-independent expression of auxin responsive genes. The caulonemal differentiation response to auxin is specifically dependent on the function of several bHLH transcription factors: PpRSL1 and 2 (Jang and Dolan, 2011), and PpLRL1 and 2 (Tam et al., 2015), all four being transcriptionally induced by auxin. Auxin signaling also leads to AP2-type transcription factors gene expression (*PpAPB*), required for apical stem cell genesis prior gametophore bud formation (Aoyama et al., 2012). Auxin-induced PpAPBs act synergistically with cytokinins to enable bud establishment, while PpAPB4 is suggested to repress auxin biosynthesis (Aoyama et al., 2012). Hence, PpAPBs constitute a major crosstalk node between cytokinins and auxin ensuring finetuned regulation of the control of bud induction. Thanks to the interplay between PpIAAs and PpARFs, auxin can have different effects on different cell types and/or at different doses, partly explained by the induction of different transcriptional responses (Lavy et al., 2016). Cell susceptibility to auxin in *P. patens* is moreover regulated through an expression gradient of negative PpARFs, owing to PpARF transcripts processing consequently to TAS3 tasiRNAs action, generated notably from the action of miR390, similarly to what is known in Angiosperms (Axtell et al., 2007; Playskin et al., 2016). This mechanism enables regulation of auxin signaling independently from auxin levels and could potentially be more sensitive to the internal state of the cell.

Auxin signaling evolution has recently been reviewed (Paponov et al., 2009; Kato et al., 2017). All constituents of the canonical nuclear signaling pathway are conserved in bryophytes (*P. patens* and *M. polymorpha*), but are not found in algae genomes, even though algae do display responses to auxin. Therefore, this pathway originated in land

plants ancestry. The ancestral effect of auxin is perhaps the induction of cell elongation, which is observed in all land plants. The dependence of moss rhizoid development on auxin signaling probably represents an ancient role given the involvement of auxin in rhizoid development in many earlier-diverging Streptophyte plants, including *Chara* and liverworts (Prigge et al., 2010). On the contrary, the role of this hormone in caulonemal differentiation in mosses could be specific to mosses, and even to the most widely represented bryopsids mosses, as other Bryophytes and early diverging mosses like *Takakia* do not display comparable *protonema* development (Prigge et al., 2010).

CYTOKININS

Natural cytokinins are adenine derivatives with a substitution on N⁶, best known in vascular plants for their promoting effect on cell division, also demonstrated in mosses (Szweykowska et al., 1971; Szweykowska and Korcz, 1972; Szweykowska et al., 1972) (Figure II-1). The structure of the substituent on N^6 is distinctive of the cytokinin's type: isoprenoid (iP type) or aromatic (kinetin type). There is an ongoing controversy about which cytokinins are majorly produced by *P. patens*. Still, it has been shown that most known types of cytokinins are produced in *P. patens*, among which N⁶-(Δ^2 -isopentenyl)-adenine (iP) (Wang et al., 1980; von Schwartzenberg et al., 2007), zeatins, aromatic cytokinins (notably benzyl-adenine (BA)), as well as derived ribosides and O-glucosides (von Schwartzenberg et al., 2007). Cytokinins produced by P. patens are secreted into the medium (Schumaker and Dietrich, 1998; von Schwartzenberg et al., 2004, 2007), but selectively: aromatic cytokinins are not secreted while iP and derivatives are the major cytokinins found in the medium. In flowering plants, two pathways enable iP biosynthesis, relying on different IPT (isopentenyl transferases) enzymes: adenylate-IPT and tRNA-IPT, which catalyse the first and limiting step of the pathway (see the review by Spíchal, 2012). Cytokinins biosynthesis in P. patens relies solely on the tRNAisopentenylation pathway, where isopentenylation and following tRNA degradation liberates cytokinin nucleotides during RNA translation (Yevdakova and von Schwartzenberg, 2007; Frébort et al., 2011). Cytokinin inactivation might be achieved in *P. patens via* phosphorylation of isopentenyl-adenosine (iPR) into isopentenyl-adenosine monophosphate (iPRMP). Indeed, *P. patens* possesses a single adenosine kinase (ADK), expressed in *chloronema*, which can produce iPRMP from iPR in vitro (von Schwartzenberg et al., 1998). On another note, cytokinin degradation is enabled by the activity of CKX enzymes (cytokinin oxidases), which predictably have a preferential activity on cis-zeatin (von Schwartzenberg et al., 2007). Frébort et al. (2011) reported 6 PpCKX genes, all encoding enzymes with conserved catalytic and ligand binding domains (Hyoung et al., 2020). Interestingly, these proteins are predicted to localize in diverse subcellular compartments, and even the extracellular space, perhaps hinting at slightly different function, although these predictions are not consensual (Gu et al., 2010; Hyoung et al., 2020).

Early studies in mosses revealed a positive role of cytokinins on bud induction and subsequent maintaining of bud identity (Gorton and Eakin, 1957; Brandes and Kende, 1968; Christianson, 1998). In *P. patens* also, cytokinins induce bud formation at low concentrations (Bopp, 1968; Ashton et al., 1979a; Wang et al., 1981) (Figure II-2). Interestingly, von Schwartzenberg *et al.* (2007) discovered that extracellular iP and iPR were responsible for cytokinin-induced bud formation, rather than intracellular cytokinins as was previously suggested by Wang *et al.* (1981). At first, cytokinins rapidly induce an increase in intracellular calcium ion levels, which mediates induction of asymmetric cell division (Saunders, 1986). Earlier, Szweykowska and colleagues (Szweykowska et al., 1971; Szweykowska and Korcz,

1972; Szweykowska et al., 1972) had suggested that an increase of cell divisions is not the main specifier of bud induction. Thus, these two effects of cytokinins can be dissociated. Then, Doonan et al. showed that microtubules are diffusely organized in bud initials compared to side branches initials (Doonan et al., 1987). They hence raised the interesting hypothesis that cytokinins rather promote bud formation by modifying the direction of cell divisions, instead of increasing cell division rates. This hypothesis conveniently explains another effect of cytokinins that is the induction of *chloronema* branching (Thelander et al., 2005). Still, the cell division induction by cytokinins is more directly linked to other effects of cytokinins, notably the promotion of protoplasts' regenerative ability (von Schwartzenberg et al., 2007) and the induction of meristematic cell formation and proliferation along the gametophore axis, leading to increase gametophore branching (Coudert et al., 2015). Moreover, exogenous cytokinins treatment was shown to elicit the formation of ectopic apical meristems on gametophores and to induce cell divisions in phyllids (Cammarata et al., 2019). Another evidence of this is the induction of callus-like buds as a presumably toxic effect of cytokinins (Decker et al., 2006). Additionally, cytokinins inhibit the formation of rhizoids (Ashton et al., 1979a; Hyoung et al., 2020) and growth of the stems (Ashton et al., 1979a). Reski and Abel (1985) suggested that chloronema and caulonema cells have a different susceptibility to cytokinins, P. patens caulonema being sensitive only to higher doses of iP for bud induction. Buds typically develop from *caulonema* but the experiments from Reski and Abel (1985) showed that both *caulonema* cells and chloronema cells are competent for cytokinin-induced bud formation (Reski and Abel, 1985). Overall, cytokinins favor the development of primary *chloronema* and buds, both chlorophyll-rich tissues. This is consistent with the findings that cytokinins can act alongside light to boost plastids metabolism, notably increasing expression of plastid polypeptides as well as *rbcL* transcript levels (Reski et al., 1991). Moreover, cytokinins were reported to induce chloroplast division in protonema (Abel et al., 1989; Reutter et al., 1998). Von Schwartzenberg also communicated on a role of cytokinins in repressing brachycytes and *tmema* development (von Schwartzenberg, 2018). Brachycytes are small round, thick-walled cells, where vacuolation and cell expansion is suppressed (they are also called brood cells) and *tmema* cells are the accompanying empty, thin-walled cells, enabling dispersal of mature brachycytes after programmed cell death (Goode et al., 1993a, 1993b). Finally, more recently, cytokinins were shown to be required for reproductive organs development and spore production (von Schwartzenberg et al., 2016; Hyoung et al., 2020). Cytokinins are active at very low concentrations relative to other phytohormones (such as ABA): 1µM BA was reported as being enough to trigger a toxic growth-inhibiting effect, possibly stemming from an increase in senescence and PCD (Thelander et al., 2005; von Schwartzenberg et al., 2016).

As underlined by Pils and Heyl, the cytokinin signaling pathway in plants is very alike a bacterial twocomponent system of signal transduction, using successive phosphorylations to finally trigger changes in target genes' expression (Pils and Heyl, 2009). Sequencing of *P. patens* genome (Rensing et al., 2008) indicated that homologs of cytokinin signaling pathway components, as CHASE-domain receptors, as well as type A and type B ARRs are present (Figure II-3). This was further investigated by Ishida and colleagues in 2010: *P. patens* has 3 *PpHK* genes among which one putative cytokinin receptor *PpHK4b* (most similar to *AtCRE1*, one of the cytokinin receptor from *Arabidopsis thaliana*)(Ishida et al., 2010). When expressed in *Escherichia coli*, the PpHK4b protein is indeed able to sense cytokinins. By looking for *P. patens* and *F. hygrometrica* genes encoding putative proteins where a histidine kinase domain is coupled with a CHASE domain and a RR domain, Gruhn *et al.* found 3 "canonical" *PpCHK* (1, 2, 3, CHK stands for CHASE-domain containing Histidine Kinase) that group with Angiosperms cytokinin receptors (likely corresponding to the 3 *PpHK* genes from Ishida *et al.* 2010) and 8 "divergent" *PpCHK* (4 to 11) that belong to a Bryophytes specific CHK clade (Gruhn et al., 2014). They selected *PpCHK4*, which is expressed both in *P. patens* and in F. hygrometrica, for functional characterization. They demonstrated in vitro the ability of the PpCHK4 protein to bind tZ but also to transduce a signal via phospho-relay from tZ, cZ, iP and BA. Thus, at least two out of the 11 PpCHK are serious cytokinin receptors candidates. The 3 canonical CHK genes from P. patens were functionally characterized only recently (von Schwartzenberg et al., 2016) and for the first time their function was actually directly assessed in *planta* thanks to characterization of all combinations of *Ppchk1-3* loss-of-function mutants. Their conclusions were that: (1) the three receptors mediate a cytokinin-dependent signal independently from each other; (2) PpCHK3 has a minor role in cytokinin-induced bud formation; (3) PpCHK1 and PpCHK2 play a major role in triggering this developmental process, PpCHK2 responding preferentially to iP, while CHK1 has a broader ligand range; (4) divergent PpCHK alone cannot ensure cytokinin response, at least for bud induction in protonema, since loss of function of PpCHK1 to PpCHK3 was sufficient for complete cytokinin insensitivity. Moving downwards the signaling pathway, Gruhn and colleagues (2014) found only two putative *PpHPt* genes where the histidine kinase domain and the conserved phosphorylation target histidine residue are both present, much less than the 6 genes reported by Ishida et al. (2010). On the other hand, they found more RR: 7 PpRRA and 5 PpRRB, which all retain the conserved aspartate residue necessary for phosphotransfer. Additionally, 3 PpRRC and 4 PpPRR genes are present in P. patens genome, but they are probably not involved in cytokinin signaling. However, these genes have not been functionally characterized yet. Recently, a possible negative feedback mechanism on cytokinin signaling was hinted at (von Schwartzenberg et al., 2016). As a matter of facts, the Ppchk1-3 mutant has elevated PpCHK2/3 transcript levels, suggesting that cytokinin perception inhibits expression of genes encoding cytokinin receptors. On the other hand, there is probably no such feedback on cytokinin biosynthesis, as the *Ppchk123* cytokinin-insensitive mutant produces similar amounts and types of cytokinins as the WT *P. patens* (von Schwartzenberg et al., 2016).

Analysis of genes associated with cytokinin biosynthesis and metabolism (Frébort et al., 2011; Spíchal, 2012) and signaling (Pils and Heyl, 2009; Gruhn et al., 2014) show that all protein domains and enzymatic activities needed were already present in bacteria and were seemingly acquired by the plant lineage by gene transfer. Then, across plant evolution, these functional domains were assembled in novel ways and eventually led to a functional signal transduction mechanism in response to the phytohormones cytokinins.

ABSCISSIC ACID

ABA is produced from carotenoids following abiotic stress in flowering plants (Figure II-1). Aside from promoting tolerance to said abiotic stress, ABA is known for regulating several developmental processes, as seed germination and root growth. *P. patens* constitutively accumulates ABA in its *protonema*, and ABA levels increase approximately 3-fold under hyperosmotic stress (Minami et al., 2006; Takezawa et al., 2015). A 10 µM ABA treatment corresponds to the endogenous levels of ABA produced by WT *P. patens* under abiotic stress and has been shown to induce a molecular response but little phenotypic changes (Arif et al., 2019). To our knowledge, only Takezawa and colleagues (2015) characterized an ABA biosynthesis gene: *PpABA1*. This gene encodes a zeaxanthin epoxidase (ZEP), likely responsible for the first step of ABA biosynthesis in plastids. The *Ppaba1* loss of function mutant does not synthesize ABA anymore, so this epoxides pathway is likely the only one leading to ABA production. Recently, Arif *et*

al. (2019) suggested the involvement of two *PpNCEDs* (9-*cis* epoxy-carotenoid dioxygenases, which homologs in Angiosperms cleave 9-*cis* xanthophylls to xanthoxin in plastids) in ABA biosynthesis.

Abscisic acid (ABA) is accumulated in slowly drying tissues of Funaria hygrometrica (Lankester, 1991), which can, after rehydration, then survive a rapid dehydration. ABA treatment of protonema enables tolerance to rapid drying, which is not possible in the absence of treatment. Thus, the induction of drought tolerance in this moss is mediated by ABA. ABA does not seem to act by preventing water loss but rather by stimulating the synthesis of protective proteins, since cycloheximide prevents ABA-mediated tolerance. These proteins were later shown to be dehydrins, some belonging to the LEA (Late Embryogenesis Abundant) superfamily (Bopp and Werner, 1993; Oliver et al., 2005). Specific mechanism of LEA action is unknown, but they likely act in stabilization and reconstitution (both during rehydration) of membranes (Oliver et al., 2005). ABA treatment also grants freezing tolerance in P. patens (Decker et al., 2006; Komatsu et al., 2009), accompanied with alterations of organelle morphology and accumulation of free soluble sugars (Komatsu et al., 2013). Komatsu et al. (2013) also suggested that ABA grants tolerance to high salinity. This implies a common ABA-mediated response to drought, freezing and osmotic stresses. Indeed, by comparing gene expression after NaCl and ABA treatment, Richardt et al. showed that both signals lead to overlapping expression profiles and that NaCl treatment induces the up regulation of ABA (putative at the time) biosynthesis genes (Richardt et al., 2010). Thus, ABA likely also mediates salt responses in P. patens (Qudeimat et al., 2008; Richardt et al., 2010). In *P. patens*, treatment with exogenous ABA causes a dramatic reduction of growth rate, leading to dwarf plants with a somehow twisted protonema. This tremendous change in morphology is caused by ABA-induced differentiation of chloronema cells into brachycytes and accompanying tmema cells (Goode et al., 1993a; Decker et al., 2006; Arif et al., 2019). Development of these specific cell types could stem from the earlier promoting effect of ABA on perpendicular cell-divisions in protonema filaments (Sakata et al., 2009), which suggests that ABA might regulate the cell cycle. ABA also apparently restricts the height of gametophores stems, which has been interpreted as an efficient mean of keeping a sufficient moisture level of the plant by keeping close to the substrate (Sakata et al., 2009; Komatsu et al., 2013). The same study from Sakata and colleagues (2009) additionally suggests that ABA induces archegonia production and thus increases the number of sporophytes, sometimes leading to multiples sporophytes per apex. But ABA also has an effect earlier, inhibiting gametophore bud formation (Christianson, 2000, see the following part on crosstalks) (Figure II-2). It has been shown this year that ABA restricts macromolecular trafficking between protonemal cells in *P. patens* (Tomoi et al., 2020), in a way seemingly independent from callose deposition. This might be linked to the cell-wall thickening preconditioning brachycytes differentiation. Fitting with its classification as a stress hormone, ABA has a dosedependent inhibiting effect on *P. patens* spore germination, and inhibition of ABA biosynthesis with norflurazon has the same effect (Moody et al., 2016; Vesty et al., 2016).

While Saruhashi *et al.* have reported the identification of four PYR/PYL/RCAR (pyrabactin resistance 1like/regulatory component of ABA receptor) homologs in *P. patens*' genome (Saruhashi et al., 2015), ABA perception by PYL/PYR/RCAR receptors or other unknown proteins has not been yet experimentally investigated. On the other hand, most of the following steps of the ABA signaling pathway in *P. patens* have been extensively investigated (Figure II-3). The first level of ABA signaling that was studied in *P. patens* is ABA-dependent transcriptional regulation via ABI3 (ABA Insensitive 3) transcription factors. *P. patens* genome encodes three ABI3 homologs: PpABI3A, B and C (Marella et al., 2006). PpABI3A, the most potent inducer of *PpLEA1* expression out of the three PpABI3s (Kamisugi and Cuming, 2005), is indeed a nuclear protein, hinting again at a role in transcriptional regulation (Marella et al., 2006). Sakata et al. reported that induction of gene expression by PpABI3A relies on direct binding to RY-element in promoters (Sakata et al., 2010). Dozens of genes encoding transcription-associated proteins are differentially expressed after ABA treatment, indicating that response to ABA has a dramatic effect on transcription (Richardt et al., 2010; Arif et al., 2019). Dependence of a gene expression on ABA in P. patens correlates well with the presence of ABRE and CE1 elements in promoter regions notably for genes encoding ABI3, bZIP and AP2 transcription factors (Oudeimat et al., 2008; Richardt et al., 2010; Timmerhaus et al., 2011). ABA-induced genes in P. patens are mostly involved in several stress response pathways, indicating overlapping pathways in the control of stress-responsive genes (Decker et al., 2006). For instance, *PpLEA1* transcripts accumulate in response to both ABA and osmotic stress (Decker et al., 2006). This induction is permitted by the ACGT core motif of the ABRE (ABA-responsive element) contained in flowering plants LEA genes' promoters (Kamisugi and Cuming, 2005). PpLEA proteins are hence accumulated in response to ABA (Komatsu et al., 2013). Concomitantly, two PP2C (Protein Phosphatase 2C) genes involved in ABA signaling in P. patens were characterized: *PpABI1A* and *PpABI1B* (Komatsu et al., 2009; Sakata et al., 2009; Komatsu et al., 2013). Among these two ubiquitously expressed genes, *PpABI1A* is quickly and durably induced by ABA treatment, osmotic stress and cold (Sakata et al., 2009). Simple *Ppabil* loss of function mutants are hypersensitive to ABA treatment but display a WT phenotype in control conditions, while the double *Ppabila/b* mutant has a slow growth and spontaneously differentiates brachycytes, thus displaying a constitutive response to ABA (Komatsu et al., 2013). Therefore, PpABI1A/B act as negative regulators of ABA signaling acting upstream of PpABI3A-induced PpLEA1 expression. Unexpectedly, bud formation was not impacted in the *Ppabi1a/b* double mutant. Therefore, *PpABI1* genes are not involved in all responses to ABA, but they do limit constitutive tolerance of protonema to diverse water-associated stresses when not enough ABA is present. However, while PpABI1s play a major role in regulating the transcriptional response to ABA (~65%), they are not responsible for all aspects of it (Komatsu et al., 2013). Indeed, ABA treatment induces kinase activity of PpSnRK2 enzymes (Komatsu et al., 2013; Saruhashi et al., 2015; Bressendorff et al., 2016; Shinozawa et al., 2019) in a PpABIIA/B-independent manner (Amagai et al., 2018). Since P. patens possesses 49 other PP2C genes, phylogenetically more distant from Angiosperms ABI1s (Sakata et al., 2009), a possible explanation is that PpABI1A/Bindependent PpSnRK2-dependent ABA signaling relies on these other PP2Cs. However, another player was identified more recently (Saruhashi et al., 2015; Stevenson et al., 2016): PpARK/ANR (ABA and abiotic stress responsive Raflike Kinase/ABA Non-Responsive). This group B3 Raf-like MAP kinase kinase kinase (B3-MAPKKK) is a major positive regulator of ABA-responsive gene expression in *P. patens*. Interestingly, this gene is actually identical to the *PpCTR1L* protein acting in ethylene signaling, characterized in 2015 by Yasumura and colleagues (see the following part on ethylene (Yasumura et al., 2015)). In order to simplify our writing, this gene will be consistently noted as *PpARK* in this review. PpARK is necessary for PpSnRK2 ABA-induced activity, suggesting it acts upstream of these kinases in the ABA signaling pathway. PpARK colocalizes with the ABA-activated PpSnRK2B in the cytosol, where it phosphorylates and activates PpSnRK2B (Shinozawa et al., 2019). PpARK is itself activated by phosphorylation in response to ABA, but the enzyme responsible for this modification is unknown. Interestingly, characterization of the Ppark loss-of-function mutant (AR7) implies that PpARK is also involved in signaling of ABA-independent hyperosmotic stress signals. Four subclass III SnRK2 are present in P. patens genome (Saruhashi et al., 2015), which homologs in flowering plants are involved in ABA signaling and are targets of ABI1 PP2Cs repressive activity. Loss of function of these genes results in variable levels of ABA insensitivity, ranging from loss of sporophyte stomata ABA

sensitivity in the simple *Ppost1-1/Ppsnrk2a* mutant (Chater et al., 2011) to complete ABA-insensitivity and hypersensitivity to diverse stresses (freezing, desiccation and hyperosmosis) in the quadruple *Ppsnrk2a/b/c/d* mutant (Shinozawa et al., 2019). Hence, PpSnRK2 activity is absolutely necessary for ABA-associated responses to stress in P. patens, mediating the induction of PpLEA genes' expression and accumulation of PpLEA proteins. Gene expression analyses showed that, while a majority of osmostress-responsive genes overlapped with ABA-responsive genes, there was a partial independence between these two transcriptional programs (Stevenson et al., 2016; Shinozawa et al., 2019). The study of Shinozawa et al. (2019) moreover led to the identification of SAGs (SnRK2-regulated and ABAupregulated Genes), among which most ARK-regulated genes were retrieved. Hence, it underlines the major role of the ABA/ARK/SnRK2 module in the regulation of ABA-responsive gene expression. Recently, Amagai and colleagues used a phosphoproteomics approach to identify PpABI1A/B-dependent and PpARK-dependent phosphopeptides (Amagai et al., 2018). They found that, whereas most PpABI1A/B-dependent phosphopeptides are regulated in an ABAindependent manner, the majority of PpARK-dependent phosphopeptides are regulated in an ABA-dependent manner (Komatsu et al., 2013; Saruhashi et al., 2015). Moreover, an alternative SnRK2-independent phosphorylation cascade in response to ABA appears to exist, as loss of function of the 4 PpSnRK2 genes (QKO mutant) prevented phosphorylation of only a portion of ABA targets (Shinozawa et al., 2019). Nonetheless, 24 proteins are common targets of PpARK and PpSnRK2s-dependent phosphorylation cascades, among which an ABA-responsive bZIP transcription factor (ABF)-related protein was found (Amagai et al., 2018; Shinozawa et al., 2019). This means PpARK and PpSnRK2 together regulate ABA signaling at least partially through phosphorylation of PpABFs. Ultimately, ABA signaling leads mainly to upregulation of gene expression (almost 600 genes), with most genes being either late responding or stably induced from earlier time-points (Arif et al., 2019). Amongst them, cell wall related genes and programmed cell death associated genes are found, which is undoubtedly linked respectively to the cell wall thickening of brachycytes and to the formation of *tmema* cells. On the other hand, ABA has a relatively modest effect on the proteome of *P. patens*, with only 65 proteins being impacted by a long-term ABA treatment (Wang et al., 2010). Nonetheless, it has been reported that ABA treatment induces an overall reduction in secreted proteins, notably for proteins regulating cell wall composition, again pointing to a role in membrane remodeling (Decker et al., 2006).

Interestingly, it was shown that the *PpNCED1* gene expression is lower in *Ppaba1* compared to WT, suggesting the existence of a positive feedback mechanism on ABA biosynthesis (Takezawa et al., 2015). Accordingly, Arif and colleagues found that 2 *PpNCEDs*, as well as almost all genes known to be involved in ABA-dependent signaling are upregulated after an ABA treatment (Arif et al., 2019). Therefore, it appears not only ABA biosynthesis but also ABA signaling is subjected to such a positive feedback.

Comparison of ABA signaling pathways between Angiosperms and Bryophytes implies that ABA has been ancestrally used as a stress hormone in land plants and that the ABA signal was transduced using the same proteins (Wang et al., 2015a). However, ABA signaling is not totally the same in *P. patens* and Angiosperms, notably at the level of ABA regulated gene expression, where the targets are quite different, as well as the interplay with ethylene using ARK (see following part on crosstalks). This could very well result from a divergent evolution specific to Bryophytes or even to mosses.



Figure II-2 - Principal hormone effects on P. patens organs and tissues. (A): Sexual organs; (B):Spore; (C): Bud; (D): Protonemal filaments, chloronema (ch) and caulonema (ca); (E): Phyllids; (F) : Branched gametophore and sporophytes (s); (G):Gametophores (g) and rhizoïds (r). Arrows indicate positive effects, dashes negative effects. Dotted lines in (E) symbolizes an effect on cell division, while square bracket an effect on cell length. Square bracket in (G) symbolizes an effect on gametophore stem length. Thickness of the arrows reflects number of experimental evidences.

ETHYLENE

Ethylene acts as a major stress hormone in flowering plants, notably involved in resistance to flooding, but also regulates several aspects of development such as floral senescence, fruit ripening and abscission of leaves. In mosses, gaseous ethylene is most probably produced via 1-aminocyclopropane-1-carboxylic acid (ACC) and not via possible precursors such as 2-ketoglutarate or glutamate, but the exact biosynthetic pathway remains to be demonstrated (Rohwer and Bopp, 1985). Existence of such an ACC-based biosynthesis pathway is also suggested by the finding of two putative genes encoding ACC-synthases in *P. patens* genome (Rensing et al., 2008).

Very few studies have focused on the effect of ethylene and its precursor ACC in mosses. The earliest report is one by Sakakibara *et al.* (2003) focusing on rhizoid development. This study concluded that ACC does not increase the number of rhizoids, although both ACC and ethylene induce root hair formation in Angiosperms. However, they did find that ACC treatment caused immediate senescence (Sakakibara et al., 2003). Almost ten years later, it was shown that ethylene treatment replicates submergence response in *P.patens* (Yasumura et al., 2012), characterized by increased *protonema* extension via *caulonema* growth and a more distal development pattern of gametophores, with them developing mainly at the periphery of the *protonema*. Ethylene promotes gametophore elongation and permits more apical rhizoid development on gametophores, belying previous reports (Yasumura et al., 2015) (Figure II-2). Yasumura and colleagues concluded that the role of ethylene relies primarily on the regulation of water status, working partially in opposition to ABA (Yasumura et al., 2012)(see the last part on crosstalks). Exogenous ACC was also shown to inhibit spore germination, although it is more likely ethylene produced from this ACC *in planta* that holds this effect, since cotreatment with ACC-oxidase inhibitor amino-isobutyric acid has no more impact on spore germination (Vesty et al., 2016).

P. patens genome encodes proteins resembling angiosperm ethylene signaling components (Rensing et al., 2008). Evidence for ethylene binding in P. patens has been reported almost fifteen years ago (Wang et al., 2006), and legitimate ethylene receptor candidates were identified in 2012 (Figure II-3). Among the seven ETR genes in P. patens, *PpETR1*, 3, 6 and 7 are phylogenetically closer to functionally characterized ethylene receptors from flowering plants and are therefore considered as best candidates (Yasumura et al., 2012). Still, all 7 predicted PpETR proteins contain a conserved histidine kinase domain and C-terminal regulator domain. Yasumura and colleagues focused on PpETR7 for functional characterization: Overexpression of a mutated version in the putative N-terminal ethylene-binding domain (*Ppetr7-1*) leads to ethylene-insensitivity, while overexpression of the WT version results in WT, submergence-like response (Yasumura et al., 2012). This shows that PpETR7 is likely a functional ethylene receptor and that it plays a major role in ethylene perception amongst PpETRs. It also proves that the mutated version has a dominant effect over the WT copy, suggesting the involvement of PpETR oligomers in perception. Moreover, the higher proportion of caulonema and shorter branches of the Ppetr7-1 plants is phenocopied by 1-MCP (1-methylcyclopropene, an inhibitor of ETR ethylene binding) application on WT P. patens. Ishida and colleagues also previously reported ethylene binding by the PpETR1c homolog in vitro (we could not retrace whether this protein is PpETR7), which inhibits the histidine kinase activity of the receptor (Ishida et al., 2010). This step occurs on the endoplasmic reticulum membrane in Angiosperms, but its localization has not been investigated yet in mosses. Possibly transducing the ethylene signal downstream of perception by ETRs are a lone CTR1 (Constitutive Triple Response 1) homolog (PpCTR1L/PpARK, cf. previous part on ABA), two EIN3/EIL1 (Ethylene Insensitive 3/EIN3-like 1) and dozens of ERF/EREBP (Ethylene Response Factors/Ethylene-Responsive Element (ERE)-Binding Proteins) transcription factors (Yasumura et al., 2012). Further inspection of *P. patens* EIN3 homologs revealed that both PpEIN3a and b do contain the EIN3 DNA-binding domain and could thus be functional transcription factors (Chang et al., 2013). Similarly, all PpERFs contain the AP2/ERF domain granting DNA binding ability to their Angiosperms' counterparts. In Angiosperms, *PpERF* expression is typically induced by EIN3 binding to their promoter region. In *P. patens*, *PpERFa* is repressed and *PpERFb* is slightly upregulated by ethylene (Yasumura et al., 2012), in a PpETR7-dependent manner. However, the involvement of PpEIN3a/b in this regulation has not been explored. Yasumura and colleagues further demonstrated that the PpCTR1L/PpARK protein does bind to PpETR7 in yeast two hybrid experiments, so it should logically be involved in ethylene signaling (Yasumura et al., 2015). The *Ppctr11/Ppark* mutant is indeed insensitive to exogenous ethylene and displays a constitutive ethylene response phenotype, showing PpCTR1L/PpARK is very likely a negative regulator of ethylene signaling. Moreover, the *Ppctr11/Ppark* mutation was determined to be epistatic to *Ppetr7-1* overexpression, proving that PpCTR1L/PpARK acts downstream of PpETR7 in the ethylene signaling cascade. However, fine characterization of the *Ppctr1l/Ppark Ppetr7-1* line suggested that PpETR7 might induce some aspects of the ethylene response independently from PpCTR1L/PpARK (Yasumura et al., 2015). These other pathways remain to be discovered. In Angiosperms, CTR1 typically phosphorylate the EIN2 protein, the master regulator of ethylene signaling, that will transduce the signal to the nucleus. To date, no study has focused on EIN2 homologues of *P. patens*, although there are at least two of them (Wang et al., 2015a).

Although ethylene's use as a phytohormone appears quite ancestral and relies on a phospho-transfer signaling cascade reminiscent of the bacterial two component system (much like cytokinins), not much is known about ethylene signaling in mosses. The role of the bifunctional PpCTR1L/PpARK homolog is very puzzling and could either represent the ancestral state of the ethylene/ABA interplay or be the product of a moss specific evolutionary route.

JASMONIC ACID and OPDA

In plants, one major class of defense hormone is composed of plant-specific oxylipins collectively known as jasmonates (Figure II-1). In Angiosperms, jasmonates are also critical for fertility and reproduction. Production of oxylipins is very conserved across evolution, between plants and animals (Ponce de León et al., 2015). However, the presence of jasmonates *per se* (jasmonic acid itself or its derivates) in mosses is the subject of ongoing debate. In 2009 two research teams reported the synthesis of jasmonic acid in *P. patens*, furthermore in higher amounts than in flowering plants (Oliver et al., 2009; Bandara et al., 2009). However, recent profiles of produced oxylipins and a study of oxylipins biosynthesis genes in *P. patens* both point to oxylipins not being further metabolized into jasmonates in this species (Senger et al., 2005; Stumpe et al., 2006; Chico et al., 2008). Nevertheless, all studies agree on the production of the jasmonate precursor 12-oxo-phytodienoic acid (OPDA) in *P. patens*. Oxylipins and thus jasmonates biosynthesis starts with the release of precursor fatty acids from membrane glycerolipids. In most plants, this fatty acid is almost invariably α -linolenic acid (Wichard et al., 2005; Anterola et al., 2009a). These free fatty acids are then oxidized into hydroperoxides by LOX enzymes (lipoxygenases). Among the eight *LOX* genes found in *P. patens*, seven encode catalytically active PpLOX enzymes with different substrate specificity. PpLOX1/2 predominant action on arachidonic

acid gives rise to 12S-hydroperoxy eicosatetraenoic acid (12-HPETE), hence starting the eicosanoid pathway, while PpLOX3-7 selective action on α -linolenic acid produces 13S-hydroperoxy octadecatrienoic acid (13-HPOTE), starting the octadecanoid pathway (Wichard et al., 2005; Anterola et al., 2009a). Following PpLOX action, two putative allene oxide synthases (AOS, OPDA biosynthesis enzymes) could follow up: PpAOS1 and PpAOS2 (Bandara et al., 2009), *PpAOS1* being more highly expressed. Recombinant PpAOS1 protein can indeed produce racemic OPDA from 13-HPOTE. PpAOS1 can also use hydroperoxy-fatty acid substrates from the eicosanoid pathway, such as 12-HPETE, as substrates, which is the main activity of PpAOS2 (Scholz et al., 2012). The allene oxides produced via AOS activity are very unstable and readily non-enzymatically degraded into racemic OPDA (Bandara et al., 2009). However, only the cis-(+)-OPDA enantiomer can give rise to natural jasmonates. The proper enantiomeric structure of OPDA is set by AOC enzymes (allene oxide cyclases) which activity is certainly temporally and spatially coupled with AOS in order to protect allene oxides from spontaneous hydrolysis (Scholz et al., 2012). There are three *PpAOC* genes in *P. patens* and all three have been shown to produce cis-(+)-OPDA from 13-HPOTE in vitro, given an AOS enzyme is provided as a helper (Stumpe et al., 2010; Hashimoto et al., 2011). Interestingly, the seven active PpLOXs and the three PpAOCs collectively localize in plastids (Stumpe et al., 2010; Hashimoto et al., 2011) along with PpAOS2 (Scholz et al., 2012), whereas PpAOS1 is cytosolic (Scholz et al., 2012), suggesting the OPDA biosynthesis pathway in P. patens might involve this subcellular compartment, which is not the case in Angiosperms. In Angiosperms, OPDA is transported into peroxisomes, where it is reduced by OPDA reductases (OPR) enzymes into 3-oxo-2-(2'(Z)-pentenyl)-cyclopentane-1octanoic acid (OPC-8:0). After three cycles of β -oxidation that shorten the carboxyl side chain, jasmonic acid is obtained. P. patens possesses at least six OPR genes, and while they have not been experimentally characterized, predicted substrate affinity and phylogenetical evidence suggest that some are relevant candidates for jasmonates' biosynthesis (Breithaupt et al., 2009; Li et al., 2009). cis-(+)-OPDA is accumulated under infection with both oomycetes (Oliver et al., 2009) and fungi, where free linolenic acid levels are also increased (Ponce De León et al., 2012). Some cis-(+)-OPDA biosynthesis genes (*PpLOX6* and *PpAOS1*, *PpAOC1*) are indeed induced by fungal infection, along with a *PpOPR* gene, suggesting this homolog might be involved in the generation of defense-promoting compounds, perhaps a jasmonate (Ponce De León et al., 2012; Toshima et al., 2014).

cis-(+)-OPDA globally inhibits *P. patens* growth, by restricting *protonema* extension and rhizoid growth, but increasing rhizoid number (Figure II-2). This effect could result from a block in cell cycle progression (Ponce De León et al., 2012). According to the phenotypes of the *Ppaoc1* and *Ppaoc2* simple mutants, *cis*-(+)-OPDA (or its derivative(s)) is necessary for fertility (Stumpe et al., 2010). However, this defect in fertility is not restored by a *cis*-(+)-OPDA treatment, neither during sporophyte induction, nor on already formed capsules, leading to the hypothesis that either *cis*-(+)-OPDA is not the active molecule or that its effect takes place much earlier in development. Hence, Stumpe and colleagues concluded that *cis*-(+)-OPDA must act at the level of gametes development (Stumpe et al., 2010). Interestingly, when Scholz and colleagues characterized the *Ppaos1* and *Ppaos2* simple loss-of-function mutants, they did not observe these types of defects, suggesting *cis*-(+)-OPDA can still be synthesized in sufficient amounts if only one PpAOS enzyme is active (Scholz et al., 2012). However, induction of *cis*-(+)-OPDA accumulation by wounding was impaired in *Ppaos1*, suggesting PpAOS1 has a prevalent effect in this case (Scholz et al., 2012). *cis*-(+)-OPDA mediates defense responses against pathogenic microorganisms such as oomycetes (Oliver et al., 2009) and fungi, and even has a direct antimicrobial activity against the latter (Ponce De León et al., 2012). *cis*-(+)-OPDA accumulation triggers *PpPAL2* (phenylalanine ammonia-lyase) expression, possibly inducing the biosynthesis of phenylpropanoids

and salicylic acid as in Angiosperms (Oliver et al., 2009). Puzzlingly, even though evidence for jasmonates' production is lacking, *P. patens* is sensitive to methyl-jasmonate (MeJA), as this molecule induces *PpPAL2* (Oliver et al., 2009) and has the same effects on growth as *cis*-(+)-OPDA, albeit higher concentrations are necessary (Ponce De León et al., 2012). An effect of jasmonates as inducers of bifunctional phytoalexic and allelopathic compounds production (momilactone A and B) has also been described, albeit not in *P. patens* but in *Hypnum plumaeforme* (Kato-Noguchi et al., 2009).

To our knowledge, there is no experimental evidence of a working signaling pathway for cis-(+)-OPDA in P. patens. It has been reported that all components of the JA signaling pathway, namely COI1 (Coronatine Insensitive 1, an F-box protein that serves as the jasmonic acid receptor in Angiosperms), JAZ transcriptional corepressors (Jasmonate-ZIM-domain proteins) and NINJA transcriptional repressors (Novel Interactor of JAZ), are present in P. patens (Rensing et al., 2008; Han, 2017)(Figure II-3). P. patens possesses several homologs of COI1 (Chico et al., 2008; Han, 2017), but we could very well hypothesize that these PpCOI-like proteins recognize other oxylipins rather than jasmonic acid, notably cis-(+)-OPDA. As written before, P. patens can also respond to jasmonic acid and its methylated derivative MeJA (Ponce De León et al., 2012), even though it is supposedly unable to synthesize these molecules. This could imply that these compounds are transformed into a bioactive molecule which can be produced from cis-(+)-OPDA in P. patens, or simply mimic the endogenous ligand of P. patens, and eventually exert similar effects. The events following signal transduction have been further explored by Toshima and colleagues when they examined the effect of a cis-(+)-OPDA treatment on P. patens proteome (Toshima et al., 2014). Almost a hundred soluble proteins are impacted by this treatment, the majority being less accumulated. Notably, carbon fixation, as well as translation and amino acid synthesis, are repressed by *cis*-(+)-OPDA, which is highly coherent with *cis*-(+)-OPDA role as a stress hormone and explains how it represses growth (Toshima et al., 2014). This effect on the proteome likely prevents unnecessary energy consumption in response to adverse environmental conditions such as infection or wounding. Interestingly, the PpAOC1 enzyme itself was over-accumulated after cis-(+)-OPDA treatment, showing that a positive feedback regulation by OPDA likely takes place in P. patens. Such a mechanism is also supported by the accumulation of proteins likely involved in generation of ROS in plastids, which could thus oxidize polyunsaturated lipids in plastid membranes, and produce the free fatty acids necessary for cis-(+)-OPDA production. Toshima et al. (2014) also suggest that cis-(+)-OPDA can regulate gene expression in plastids. Last year, Fesenko and colleagues showed that MeJA treatment of P. patens protonema induces the production of new small peptides especially from chloroplastic precursor proteins, possibly by action of the ubiquitin-proteasome system, with some being released extracellularly (Fesenko et al., 2019). Certain peptides from this secretome had a bacteriostatic effect on E. coli and B. subtilis and one could induce the expression of pathogenesis-related genes in *P. patens*, hinting at a possible signaling ability (Fesenko et al., 2019).

cis-(+)-OPDA might not have been used as a hormone *per se* in ancestral plants, but rather as defense compounds against pathogens and wounding (this hypothesis is further discussed in the review by de León et al., 2015). However, the effects of this molecule in *P. patens* fertility, at micromolar doses, do suggest a hormonal role. Indeed, a derivate from this molecule (dinor OPDA) has a demonstrated hormonal role in another Bryophyte, the liverwort *Marchantia polymorpha* (Monte et al., 2018). Elucidation of a *cis*-(+)-OPDA (or derivative(s))-specific signaling pathway would definitely help determining whether this (these) molecule(s) are *bona fide* hormones in mosses.

SALICYLIC ACID

Salicylic acid (SA) is a phenolic compound (Figure II-1) acting as a major stress hormone, protecting Angiosperms against pathogens, notably by enabling pre-emptive defense reaction in cells located at a distance from the plant/pathogen interaction point (namely Systemic Acquired Resistance or SAR). SA can be produced in plants through two different pathways, both starting from chorismate: the ICS (isochorismate synthase) plastidial pathway and the PAL (phenylalanine ammonia-lyase) cytosolic pathway (see the recent review by Lefevere et al., 2020). Neither pathways have been characterized in mosses, nor possible mechanisms regulating SA catabolism, transport or storage. However, *P. patens* can produce SA (Richter et al., 2012). *P. patens* moreover possesses several *PpPAL* genes, but it is worth to remember that PAL enzymes are also involved in phenylpropanoid biosynthesis and not specific to SA biosynthesis (Oliver et al., 2009; Wolf et al., 2010).

To our knowledge, only one study by Christianson and Duffy investigated the impact of salicylates on *P. patens* development (Christianson and Duffy, 2002). They determined that both salicylic acid itself, as well as acetylsalicylic acid, can inhibit bud formation in a dose-dependent way (Figure II-2). They further established that this effect likely does not result from an impairment of cytokinin-induced bud induction as it occurs much later in bud formation and is neither explained by an inhibition of young gametophores outgrowth. Several elements suggest that SA is involved in defense promotion in *P. patens*. Firstly, Salicylic acid (SA) levels increase rapidly after fungal infection, and treatment with SA enhances transcript accumulation of one of the phenylalanine ammonia-lyase (PAL) defense genes in *P. patens* (Ponce De León et al., 2012). SA levels are increased 7-fold in a few hours following the onset of *B. cinereal* infection, suggesting that SA is synthesized and perceived during pathogen infection. In contrast, there was no induction of SA accumulation after oomycetes infection according to a study by Oliver *et al.* (2009), so the SA pathway might not be involved in defense against all microbial pathogens.

Studies about SA signaling in mosses are scarce and recent. Peng et al. identified two putative NPR (Nonexpresser of PR genes) SA receptors in P. patens genome (Peng et al., 2017), confirming previous predictions (Wang et al., 2015a). However, one of them appeared to be a pseudogene and only *PpNPR1* was characterized. It contains both conserved protein interaction domains: The N-terminal BTB/POZ domain and the central ankyrin-repeat domain, as well as a C-terminal domain with nuclear localization signals. Functional complementation of the Arabidopsis Atnpr1 mutant suggested that PpNPR1 is indeed a functional receptor of SA and is even able to properly interact with other players in the SA signaling pathway of Angiosperms. Thus, SA signaling is likely similar between Angiosperms and mosses. Nevertheless, in Angiosperms, paralogs of NPR1 called NPR3/4 act as negative regulators of response to SA. This function of NPRs does not exist in *P. patens*, as the *Atnpr3/4* mutant is not complemented by *PpNPR1* unlike *Atnpr1*. It would imply that mosses rely on other mechanisms to fine-tune the SA signaling level of activation. Other cellular events downstream of *PpNPR1* are not known yet in *P. patens* or other mosses. Whether PpNPR1 can, similarly to Angiosperms NPR proteins, interact with WRKY and TGA transcription factors in the nucleus to transduce the SA signal remains undetermined. Nevertheless, the fact that SA does rapidly induce the expression of defense genes, namely *PpPAL1* (Ponce De León et al., 2012) and *PpPR* gene(s) (Peng et al., 2017), implies that PpNPR1 is indeed interacting with (a) transcription factor(s) to induce SAR (Systemic Acquired Resistance). SA also triggers effects at the protein level, as Filippova *et al.* have shown that a SA treatment activates specific proteases to generate a pool of almost 150 new active peptides in protonema, some being secreted (Filippova et al., 2019). SA treatment notably induced proteolysis of small stress-related proteins, and overall results suggest that SA caused a significant increase in proteasome activity. However, the potential roles of these new peptides, either as signaling elements in defense, or as molecules with a direct effect against possible causes of stress, is not known.

Despite the lack of a decisive number of studies on SA biosynthesis and signaling, present evidence suggests that the use of SA as a defense hormone is ancestral to land plants. Nonetheless, further investigation might reveal some minor divergences between Bryophytes and vascular plants.

GIBBERELLINS and KAURENE Derivatives

Gibberellins are tetracyclic diterpene acids (Figure II-1) synthesized in plants but also in fungi and bacteria. In flowering plants, they are major regulators of diverse aspects of development, notably inducing the elongation of stems by triggering cell elongation, which was their first discovered role, and inducing seed germination. The first steps of gibberellic acid (GA) biosynthesis in plastids lead to the formation of the key intermediate ent-kaurene, which was shown to be also true in *P. patens* (Anterola et al., 2009b; Hayashi et al., 2010). However, moss relies on a single, bifunctional enzyme copalyl-diphosphate synthase/ent-kaurene synthase (PpCPS/KS), to catalyze the two steps necessary for ent-kaurene synthesis (Hayashi et al., 2006; Anterola et al., 2009b). This makes ent-kaurene biosynthesis in moss more alike to what has been shown in fungi (Hedden et al., 2001; Davidson et al., 2006; Ross and Reid, 2010) rather than what happens in vascular plants where the two enzymatic activities are separate. The CPS enzymatic activity of the PpCPS/KS protein converts geranyl-geranyl-diphosphate (GGDP) into ent-copalyl-diphosphate, and then the KS activity transforms the ent-copalyl-diphosphate into ent-kaurene. Then, ent-kaurene is likely oxidized into ent-kaurenoic acid, this molecule being produced at high levels in protonema (Hayashi et al., 2010). Indeed, P. patens possesses a gene that could encode a CYP450 with *ent*-kaurene oxidase activity (*PpKO/PpCYP701B1*) and could thus be able to synthesize ent-kaurenoic (Hayashi et al., 2010). Interestingly, loss-of-function of PpCPS/KS results in complete absence of ent-kaurene and ent-kaurenoic acid, thus this enzyme is responsible for the biosynthesis of the whole pool of entkaurene in P. patens (Hayashi et al., 2010). ent-kaurenoic acid is then transformed into ent-3b-hydroxy-kaurenoic acid (3OH-KA) and ent-2a-hydroxy-kaurenoic acid (2OH-KA), both being produced along protonema development (Miyazaki et al., 2018). 2OH-KA biosynthesis is provided by the activity of an *ent*-kaurenoic acid oxidase (PpKA2ox), however the enzyme enabling 3OH-KA production was not identified. Interestingly, PpKA2ox was eventually demonstrated to be an ent-kaurenoic acid inactivation enzyme, in accordance with 2OH-KA being less bioactive than 3OH-KA. Surprisingly, *PpKA2ox* transcription is decreased by *ent*-kaurenoic acid, implying that 3OH-KA accumulation is subjected to positive feedback (Miyazaki et al., 2018). GA biosynthesis steps following 3OH-KA are far less clearly established. In 2010, Hayashi and colleagues underlined that, as several "canonical" gibberellins have similar effects on P. patens as ent-kaurenoic acid, P. patens is expected be able to synthesize such compounds. But they also acknowledged that the biosynthesis steps leading to these GA, most likely needing enzymes of the CYP450 superfamily, remain to be elucidated. Very recently, a whole genome comparison study by Cannell et al. suggested that genes potentially encoding canonical gibberellins' biosynthesis enzymes (production of GA12 and all derived GA) are missing in both *P. patens* and *Sphagnum fallax* genomes, whereas they are present in other Bryophytes (Cannell et al., 2020). They hence concluded on a secondary loss of these genes along mosses evolution. However, an earlier study reported that *P. patens* possesses homologs of GA_{20} oxidase and GA_3 oxidase encoding genes, suggesting respectively GA_{20} and GA_3 (gibberellic acid) can be produced (Hirano et al., 2007). Nevertheless, GA production or GA oxidase activity could not be detected in this moss (Hirano et al., 2007).

GA₃ seems to have no effect on *P. patens* protonemal development, even at high concentrations (Vandenbussche et al., 2007). Moreover, the gibberellin biosynthesis inhibitor paclobutrazol (PAC) inhibits protonemal growth, but GA3 addition does not reverse this effect, further suggesting that *P. patens* is insensitive to this molecule (Yasumura et al., 2007). Yet, GA₃ can inhibit P. patens spores' germination (Figure II-2), although not as efficiently as ent-kaurene (Anterola et al., 2009b). Unexpectedly, an ent-kaurene biosynthesis inhibitor (AMO-1618) severely decreases germination, but its effect is alleviated by addition of *ent*-kaurene only (and not by GA₃). This further shows that the active gibberellin-like molecule is not GA₃ but likely an *ent*-kaurene derivative or *ent*-kaurene itself (Anterola et al., 2009b). Both ent-kaurene and ent-kaurenoic acid are major inducers of the transition from chloronema to caulonema, but otherwise do not seem to affect cell size and growth rate in filaments (Hayashi et al., 2010) (Figure II-2). Interestingly, GA₉-methyl ester could also promote *caulonema* differentiation, albeit being ~10 times less potent than ent-kaurenoic acid (Hayashi et al., 2010), implying that P. patens could produce active compounds similar to "canonical" GA. On another note, inhibiting synthesis of ent-kaurenoic acid from ent-kaurene with uniconazole decreased *caulonema* number in WT, and this effect is lost when *ent*-kaurenoic acid was co-applied (Hayashi et al., 2010). Therefore, it suggests that *ent*-kaurene itself is not the active compound, but rather *ent*-kaurenoic acid or a derivative, which was confirmed later as *Ppko* and *Pcps/ks* mutants display the same phenotype (lack of blue light avoidance) (Miyazaki et al., 2015). On the other hand, neither ent-kaurene or ent-kaurenoic acid appear to have a significant effect on gametophores development, according to the lack of defects of the *Ppks* mutant. Puzzlingly, the Ppks mutant has WT fertility and spore germination rate (Miyazaki et al., 2015), suggesting ent-kaurene and its derivatives are not necessary for proper reproduction, contrary to what was hypothesized before (Anterola et al., 2009b). Hayashi and colleagues also showed that AMO-1618 neither impair ent-kaurene production in the protonema, nor affect chloronema to caulonema transition (Hayashi et al., 2010). Therefore, the previously described effect of AMO-1618 on spore germination is likely imputable to another pathway rather than to an ent-kaurene response defect. This was infirmed later, as *Ppks* mutants were revealed to have a reduced germination speed compared with WT, although they eventually attained 100% germination (Vesty et al., 2016). Moreover, germination speed was reduced after application of either GA9-methyl ester or ent-kaurene, but consistently not GA3 (contradicting the previous observation of Anterola et al.). Taken together, these studies demonstrate that P. patens can respond in a similar fashion to ent-kaurenoic acid and to some other, likely derived from the former, compounds more similar to "canonical" GA such as GA₉-methyl ester. However, P. patens cannot respond to GA₃, which is the most bioactive GA is flowering plants, thus GA perception likely has a different specificity in mosses.

The gibberellin signaling pathway was extensively investigated by Hirano and colleagues (Hirano et al., 2007), who identified two genes encoding putative receptors of gibberellins in *P. patens: PpGID1L1* (GIBBERELLIN INSENSITIVE DWARF1 Like1) and *PpGID1L2* (Figure II-3). PpGID1Ls, like their Angiosperms' homologs are alphabeta-hydrolases/hormone sensitive lipases (HSL). However, contrary to Angiosperms, PpGID1Ls retain the Ser-Asp-His catalytic triad, which suggest they might still be catalytically active (Hirano et al., 2007), but this point is debated as another team found the His residue of the triad to be replaced by a tryptophan in PpGID1Ls (Vandenbussche et al.,

2007). Moreover, two residues that are known to be necessary for gibberellins perception are lost in these two proteins, thus their function as gibberellins receptors cannot be assumed. These proteins are not able to bind neither GA_4 , GA_9 , GA₁₂ nor 3-epi-GA₄ in vitro (Hirano et al., 2007), which retrospectively appears coherent with the lack of physiological response of P. patens to these molecules. ent-kaurene and ent-kaurenoic acid binding to PpGID1Ls ought to be explored, as well as with the impact of PpDELLA-Likes on this binding, since DELLAs strengthen interaction between GID1 and gibberellins in Angiosperms (Hirano et al., 2007). PpGID1L1 (PpGLP1 for Yasumura et al., 2007) could not interact with PpDELLA-L1 in a yeast two hybrid assay with or without GA₃, however it could interact with a Lycophyte DELLA even in the absence of GA₃, suggesting the lack of interaction of moss proteins stems from PpDELLA-Ls. Therefore, affinity of GID1-like proteins for DELLAs could be ancestral and has been lost in Bryophytes, while DELLAs affinity for GID1-likes would have evolved after the split of Bryophytes and vascular plants. The necessity for gibberellins to trigger this interaction would have appeared even later, after Lycophytes diverged from other vascular plants. Alternatively, this interaction might rely on other compounds, notably *ent*-kaurenoic acid, which was not tested in these studies. Three genes encoding putative F-box proteins (PpGID2L1 to 3) are found in P. patens genome (Hirano et al., 2007). Analysis of their phylogeny and domain composition however suggests that, while they likely play a role in targeting some proteins for proteasomal degradation, it is not in the context of gibberellins signaling (Hirano et al., 2007; Vandenbussche et al., 2007). Another evidence that P. patens might not be able to sense gibberellins is that the PpDELLA-L proteins do not contain a conserved DELLA motif, responsible for the gibberellins-induced interaction with GID1 and subsequent proteasomal degradation. Moreover, PpDELLA-Ls lack half of the conserved tyrosine residues known as necessary for gibberellins-induced degradation of DELLAs from A. thaliana. Despite this discrepancy, PpDELLA-Ls do possess the C-terminal GRAS domain involved in protein-protein interactions and possibly in DNA binding (Hirano et al., 2007). Also, they group with Angiosperms' DELLA proteins phylogenetically. It can be argued that the absence of strict homology of domain and the absence of residues characterized as necessary in Angiosperms do not necessarily mean that these proteins are not involved in gibberellin signaling in mosses. However, some experimental evidences go along with this hypothesis. Notably, in a yeast two hybrid experiment, PpDELLA-Ls are unable to interact with either PpGID1L, even triggered by gibberellins addition (Hirano et al., 2007). Since DELLA proteins are homologs of the other GRAS proteins SLR-like (SCARECROW-like), it can be hypothesized that PpDELLA-Ls are in fact more functionally related to SLR-likes and rather play a role in development regulation (Vandenbussche et al., 2007). The lack of developmental phenotype of the *Ppdella-l1/2* loss of function mutant, along with its WT-like sensitivity to PAC, suggests that biosynthesis of gibberellin-like molecules in P. patens is irrelevant to the function of PpDELLA-Ls (Yasumura et al., 2007). Hence, PpDELLA-Ls are not involved in the response to GA in P. patens, and they are not potent regulators of development either. When expressed in Arabidopsis roots, GFP-fused PpDELLA-L1 localizes to nuclei and its levels are not impacted by GA₃ treatment (Yasumura et al., 2007). However, its introduction apparently corrects the dwarf phenotype of the DELLA gal-3 mutant of Arabidopsis, suggesting it can properly replace GA1. It is puzzling that PpDELLA-L1 can restrain growth in Arabidopsis but not in P. patens itself. Yasumura and colleagues raise the interesting hypothesis that this phenomenon could be explained by growth controlling genes becoming DELLA responsive along evolution via changes in cis-regulatory regions. Thus, in P. patens, these genes controlling growth would not be under the transcriptional control of PpDELLA-Ls (Yasumura et al., 2007). More recently, coexpression networks involving DELLA proteins were compared between P. patens and flowering plants (A. thaliana and Solanum lycopersicon), revealing that DELLAs likely act as hubs of transcriptional regulation in *P. patens* (Briones-Moreno et al., 2017). Functional annotation of genes involved in PpDELLA-Ls networks suggest a major implication in response to stress.

Taken together, results cited before suggest that *P. patens* is unable to respond to canonical gibberellins but that the gibberellin precursor *ent*-kaurenoic acid might be relevant during early development of the gametophyte. Since some gibberellins trigger similar responses to *ent*-kaurenoic acid, it is possible that the signaling cascade involved in *P. patens* works in a way that cannot be inferred by taking the Angiosperms pathway as a basis. For instance, even if the homologs of GID and DELLA genes are actually involved in a gibberellin-signaling cascade, the exact residues involved in these proteins have evolved independently so they might be quite different from residues necessary in Angiosperms. Mosses might have secondarily lost the ability to sense gibberellins (Vandenbussche et al., 2007; Yasumura et al., 2007), as early studies reported that both a liverwort and a Charophyte can respond to gibberellins (Asprey et al., 1958; Kwiatkowska et al., 1998). This loss of response ability is relevant if we consider the possible secondary loss of canonical gibberellins' biosynthesis in mosses (Cannell et al., 2020). However, that could imply that the demonstrated effect of *ent*-kaurenoic acid and/or derived compounds is not perceived and transduced through the same pathway as canonical gibberellins.

BRASSINOSTEROIDS

Brassinosteroids (BRs) are a group of hydroxylated steroid-derived compounds (Figure II-1) undertaking very diverse roles in Angiosperms, from the promotion of senescence to ensuring pollen fertility, and from regulation of cell growth to protection of plants against abiotic stress. In contrast to auxins and GAs, there is no report yet of BRs in fungi or bacteria, thus BRs appear to be strictly plant-specific compounds (see the review by Ross and Reid, 2010). Much like jasmonates, the presence of BRs in moss is still under debate. While sterol biosynthesis upstream of BRs is highly conserved across land plants, no clear homologs of BR biosynthesis genes are found in *P. patens* genome, except DET2 (involved in campestanol (CN) generation from campesterol (CR), Cannell et al. 2020). Two cytochrome P450 enzymes, CYP710A13, and CYP710A14, were identified by Morikawa et al. (2009) as sterol C22-desaturases in P. patens. These enzymes can use β-sitosterol *in vitro* to produce stigmasterol, but they cannot use campesterol and 24-*epi*-campesterol as substrates. This evidence again indicated the presence of an entire sterol biosynthetic pathway in this moss, although the sterols produced are different from flowering plants. These two enzymes are hence unlikely to be a part of a BR biosynthesis pathway. More recently, Yokota et al. reported that P. patens possesses both CYP85 and many CYP72 enzymes, respectively required for biosynthesis and inactivation of BR. Cannell and colleagues disagreed on this point as they noted that non-seed plants lack clear homologues of these inactivating CYP450 enzymes (Yokota et al., 2017; Cannell et al., 2020). Despite these uncertainties, P. patens produces castasterone (CS), which is a bioactive BR in Angiosperms (Yokota et al., 2017). Therefore, P. patens can likely synthesize and inactivate BR, but the enzymes involved might not be close homologs to those of Angiosperms and could therefore not be recovered by BLAST.

No homolog of the BR receptor BRASSINOSTEROID INSENSITIVE 1 (BRI1) is found in *P. patens* (Rensing et al., 2008). In facts, the only partially similar sequences found lack the key extracellular domain involved with BR binding (Ross and Reid, 2010). In their review on phytohormones signaling evolution, Wang *et al.* (2015) reported that several other components of the BR signaling pathway are present in *P. patens*, notably the co-receptor BRI1-Associated
receptor Kinase (BAK1). However, this species is missing the key regulator Brassinosteroid Insensitive1 Kinase Inhibitor (BKI1), which appears to be specific to Angiosperms. Other molecular players of the BR signaling pathway are more ancient, as they are similarly found in flowering plants and in *P. patens*. This is the case for the kinase BRASSINOSTEROID INSENSITIVE 2 (BIN2) and for the transcription factor BRASSINAZOLE RESISTANT 1 (BZR1). But they have not been experimentally assigned to BR signaling in *P. patens*. Similarly, BIL4 (BZR-Insensitive Long hypocotyl 4), a seven transmembrane-domains protein similar to G protein-coupled receptors, has a homolog in *P. patens*. In Arabidopsis, this protein interacts with the BR receptor BRI1 in endosomes to prevent its relocation and degradation in vacuoles, but in *P. patens*, BIL4 function is likely independent of BR signaling (Yamagami et al., 2017). The evidence on biosynthesis, effects and signaling are thus too fragmentary to establish whether BRs are actually used as phytohormones in mosses.

Consistently, in their 2010 review, Ross and Reid emitted the hypothesis that BRs or BR-like compounds could also simply be present as specialized metabolites in mosses, rather than act as actual hormones. That could explain the lack of a specific signaling pathway in the Bryophytes species studied until now, despite their synthesizing BR-like compounds.

SIGNALING PEPTIDES

To date, sixteen families of plant signaling peptides have been identified in Angiosperms. These peptides belong to two major groups: Group I consists of cysteine-rich peptides for which proteolytic processing is not always necessary, while group II includes cysteine-poor, smaller, peptides, and must be proteolytically processed to maturity (see the review by Ghorbani, 2014). Most signaling peptides belong to group II, notably the CLE peptides (Clavata3/Endosperm Surrounding Region (ESR)-Related), which have been the focus of extensive research effort. In Angiosperms, CLE peptides have been shown to have dramatic effects on stem cells maintenance. Two classes of CLEs have been described: CLV3-like and TDIF-like (Tracheary element Differentiation Inhibitory Factor-like). The CLV3 (Clavata3) peptide hormone has been especially well characterized. CLE peptides production is initiated by the translation of a short (~200 residues) pre-propeptide containing a N-terminal secretion signal. This pre-propeptide contains the CLE domain, which is only 12-13 amino acid long. The inactive pre-propeptide must be enzymatically cut by serine- or carboxy-peptidase for the CLE peptide to be freed, although some CLE peptides need to be further post-translationally modified to become biologically active and be ultimately secreted in the extracellular space (Goad et al., 2017).

Genes encoding CLE peptides are present in *P. patens* genome, even though CLE genes are difficult to identify since only the CLE domain is usually highly conserved, and since most are lowly expressed and thus not annotated in genomes (Sawa and Tabata, 2011; Goad et al., 2017). More precisely, only 9 *PpCLE* genes were identified, but more divergent ones might have been missed (Goad et al., 2017). Further research by Whitewoods *et al.* led to the identification of only 7 non-divergent *PpCLE*, giving rise to four different peptides: PpCLE1/2/3, PpCLE4, PpCLE5/6 and PpCLE7 (named from their encoding gene(s))(Whitewoods et al., 2017). All four peptides contain most of the residues necessary for interaction with CLV1 receptor (Goad et al., 2017). *PpCLE1, PpCLE2* and *PpCLE7* are mainly expressed in gametophores, while *PpCLE6* expression is *protonema* specific. On the other hand, *PpCLE3, PpCLE4* and *PpCLE5* transcripts could not be detected (Whitewoods et al., 2018). Both studies agree that all PpCLEs belong to the

CLV3-like class. In *P. patens* as in Angiosperms, the precise enzymes involved in CLE peptides generation have not been formally identified.

CLV3 in Angiosperms can potentially be sensed via three different family of RECEPTOR-LIKE KINASES (RLK) receptors: CLV1 (Clavata1), CLV2 (Clavata2, along with the SOL2/CRN (Suppressor Of LLP1 2/Coryne) coreceptor) and RPK2 (Receptor-like Protein Kinase 2). P. patens has 2 homologs of CLV1 (PpCLV1a and PpCLV1b), as well as one of RPK2 (PpRPK2), but no CLV2 and SOL2/CRN (Sawa and Tabata, 2011; Whitewoods et al., 2018). Whitewoods et al. report the expression of all three genes in gametophores stems and phyllids. Earlier, PpCLV1a transcripts are accumulated in protonema cells around buds, while PpCLV1b and PpRPK2 accumulate in buds themselves, especially at the apex. Further confirming that CLE signaling is involved in transition to gametophore 3D growth, Whitewoods and colleagues noted that *Ppcle* knock down lines and *Ppclv1a/b* knock out lines developed far less, dwarf, gametophores. Close inspection of buds revealed misdirection of division planes in the first divisions of the buds. However, since gametophores eventually develop, the disruption likely occurs after "bud" cell fate is specified, while still taking place at the single-celled stage. In gametophores, all lines had defective phyllids development (Whitewoods et al., 2018). Moreover, *Pprpk2* and *Ppclv1a/b* lines developed calluses at the gametophore base, resulting from the activity of ectopic apical cells. Puzzlingly, exogenous application of PpCLEs also caused gametophore dwarfing and a decrease in phyllids size, but these were not the results of the same developmental arrest and meristematic over-proliferation as in *Ppcle* lines. Therefore, it could be concluded that a very narrow range in *PpcLEs* concentration must be achieved to permit WT development of gametophores. Finally, Whitewoods and colleagues demonstrated that Pprpk2 is indeed unable to respond to neither PpCLE1,2,3 nor PpCLE7, in terms of gametophore dwarfing (Whitewoods et al., 2018). Interestingly, P. patens does not respond to the TDIF peptide from Arabidopsis, while it does respond to the CLV3 peptide from this same species. This again points to mosses not using TDIF-like CLEs, which likely stems from a secondary loss in their evolutionary lineage (see the recent review by Whitewoods, 2020).

STRIGOLACTONES and **KAI2-ligand**(s)

Strigolactones (SL) are cyclic lactones originating from apocarotenoids (Figure II-1). They are the most recently established class of phytohormones and play many roles in development in Angiosperms, notably repressing axillary branching, promoting secondary growth and regulating root architecture. In addition, SL are involved in the regulation of interspecific interactions between plants and micro-organisms, of both symbiotic and pathogenic nature, and help the plant cope with several abiotic stresses (see the review by Mishra et al., 2017). Sequencing of *P. patens* genome has shown that this moss possesses homologs genes to those encoding the first enzymes of the SL biosynthetic pathway, leading to carlactone (CL, Figure II-1) production: the isomerase D27 (DWARF 27), and the CAROTENOID CLEAVAGE DIOXYGENASES CCD7 and CCD8 (Rensing et al., 2008). On the other hand, no clear CYP711A1 candidate for catalyzing the transformation of the CL precursor into SL has been identified. Recent quantifications of SL in *P. patens* revealed only the presence of CL, in accordance with the absence of CYP711A1 enzymes (Decker et al., 2017; Yoneyama et al., 2018b). However, it does not necessarily mean that no other compound with SL activity is synthesized by *P. patens*: it cannot be excluded that enzymes apart from CYP711A1 (maybe even other classes of CYP450) could use CL as a substrate. Furthermore, several compounds beside the precursor of CL can be obtained from

PpCCD7 enzymatic activity *in vitro* (Decker et al., 2017) and add a layer in the putative structural diversity of SL like compounds in *P. patens*. Decker *et al.* also confirmed PpCCD8 activity as a CL synthase *in vitro*. It is widely admitted that SL are unstable molecules and are furthermore produced at very low concentrations. These characteristics make their identification (and use) quite difficult, even in flowering plants, and is part of why there is no certainty about the exact SL molecules produced by *P. patens*. This, along with the fact that SL receptors break their substrates down into inactive molecules *in vitro* (Bürger et al., 2019), also makes enzymatic inactivation of SL dispensable, unlike for other phytohormones such as cytokinins.

In 2011, the first homolog of a SL biosynthesis gene in P. patens, PpCCD8, was functionally characterized by Proust et al. Study of the loss-of-function Ppccd8 mutant and use of the synthetic SL analog racGR24 (Figure II-1) revealed that SL in P. patens repress spore germination (Proust et al., 2011; Vesty et al., 2016). SL also repress branching of primary *chloronema*, while they do not appear to affect *caulonema* branching until 21 days after spore germination, when *Ppccd8 caulonema* filaments continue to develop secondary *chloronema* and buds at the periphery, unlike WT (Proust et al., 2011). This suggests that SL do repress *caulonema* branching at this later time point and/or at the apex of filaments (Figure II-2). Proust and colleagues suggested this effect on branching could be explained by SL inhibiting the cell division rate, at least in *chloronema*. Effects of SL on cell division and cell elongation were clarified a few year later, when closer inspection of the *Ppccd8* mutant revealed that SL likely repress cell division rate, and less potently cell elongation, in both types of protonemal filaments (Hoffmann et al., 2014). This was further confirmed by Decker and colleagues when they demonstrated that the *Ppccd7* loss of function mutant displays the same increase in filaments length, resulting of an increase in cell number, as *Ppccd8* (Decker et al., 2017). A study by Coudert *et al.* suggested that this inhibitory effect on cell division is also active in gametophores (Figure II-2), as the *Ppccd8* mutant displays strongly increased branching of gametophores at their base and this phenotype was suppressed by racGR24 (Coudert et al., 2015). This branching phenotype is reminiscent of SL insensitive mutants from Angiosperms (affecting MORE AXILLARY BRANCHES, MAX genes), suggesting that inhibition of branching, albeit involving different tissues and processes in mosses and Angiosperms, is an ancestral role of SL. Puzzlingly, SL increase rhizoid length in P. patens, which could mean that they have a positive effect on cell division and/or cell elongation in this tissue, contrary to their effect on protonema (Delaux et al., 2012). On another note, it is interesting to underline that, similarly to cytokinins, SL are liberated in the medium (Proust et al., 2011). Proust et al. suggested that SL act as bacterial quorum sensing molecules, regulating growth of close neighboring plants, as their concentration in the medium reflects plant density. Decker et al. described a protective effect in P. patens against the phytopathogenic fungi Fusarium oxysporum and Sclerotinia sclerotiorum (Decker et al., 2017). This effect is most probably explained by an enhancement of P. patens resistance, but the mechanism has not been investigated yet. Moreover, they can have an effect in protecting whole P. patens populations from pathogens, as they increase resistance of the plants (Decker et al., 2017). Furthermore, Vesty et al. suggested that this quorum sensing mechanism could be particularly of use during spore germination: plants developing form the first germinating spores would produce SL and therefore prevent germination of close spores (Vesty et al., 2016). This could alleviate intraspecific competition for resources. Taken together, these results point to SL being a major mean of shaping and organizing P. patens whole populations. P. patens responds similarly to the synthetic analog of SL racGR24 and to WT exudates, suggesting racGR24 is sensed through the same signaling cascade as compounds derived from PpCCD8 activity (Proust et al., 2011). CL itself rescues *Ppccd7* and *Ppccd8* hyper-branched phenotype (Decker et al., 2017), implying either that CL itself is the bioactive compound in *P. patens*, or that CL can

be converted to a bioactive SL *in vivo*. *P. patens* can also respond to some natural canonical SL, notably solanacol and 5-deoxystrigol (Hoffmann et al., 2014), hence this (these) CL-derived compound(s) might have a similar structure.

In Angiosperms, two different subclasses of related KAI2L (KARRIKIN INSENSITIVE 2-like) α/β -hydrolases, euKAI2 and DDK subclasses, are involved in the perception of two types of phytohormones: DDKs handle SL perception, whereas euKAI2s tackle KL (KAI2-ligand) perception (Bythell-Douglas et al., 2017). KL is a conceptual term used to refer to unknown endogenous compounds that are perceived by euKAI2s and are widely considered as a new type of phytohormones. In 2016, a study by Lopez-Obando and colleagues identified thirteen KAI2L genes in P. patens (Figure II-3). Investigation of these genes' expression and modelling of the putative proteins denoted PpKAI2L-G and -J as the best candidates for SL perception, whereas PpKAI2L-B, -C, -D and -E are closer to euKAI2 and therefore are candidate for KL perception. Karrikins are natural compounds found in fire smoke and stimulating seed germination and seedling emergence after a fire, which are usually used as KL mimics (Flematti et al., 2004). Biochemical characterization by Bürger et al. (2019) however showed that PpKAI2L-C, -D and -E cannot bind the karrikin KAR1 (Figure II-1), while PpKAI2L-H, -L and -K can. Nevertheless, it must be noted that the biological relevance of these results is for the moment lacking, especially since P. patens has been shown to be non-responsive to KAR1 (Hoffmann et al., 2014). Characterized euKAI2/DDK proteins in Angiosperms both interact with the same F-box protein MAX2 (MORE AXILLARY BRANCHES 2) and facilitate the involvement of MAX2 in a SCF E3-ligase ubiquitination complex (Yao and Waters, 2020). This complex then triggers the proteasomal degradation of proteins from the SMXL (SUPPRESSOR OF MAX2-1-like) family. In flowering plants, SMXL proteins of different groups are associated with either euKAI2-dependent signaling or DDK-dependent signaling (Yao and Waters, 2020). However, in non-seed plants such as *P. patens*, this divide in function has not been proved and phylogeny cannot predict in which pathway they might act (Moturu et al., 2018; Walker et al., 2019) (Figure II-3). In addition to this, a recent study of the sole MAX2 homolog from P. patens (Lopez-Obando et al., 2018) demonstrated that PpMAX2 is most probably not involved in SL signaling. Indeed, *Ppmax2* loss-of-function mutants have a striking developmental phenotype that is completely different from that of SL biosynthesis mutants (Ppccd7 and Ppccd8). Additionally, these Ppmax2 mutants can respond to exogenous SL, even more significantly than WT. PpMAX2 is instead involved in photomorphogenesis, which is hypothesized to be linked to KL signaling (Lopez-Obando et al., 2018). If PpMAX2 is involved in KL signaling, then this response in *P. patens* likely takes place where *PpMAX2* is expressed: *chloronema* and young *caulonema*, the basal portion of gametophores and sporophytes (Lopez-Obando et al., 2018). Given the apparent roles of PpMAX2 in promoting chloronema to caulonema transition and gametophores development, KL likely has similar roles in P. patens, maybe through an induction of cell division, contrary to SL. Going back to the SL response pathway, although signaling events downstream of perception are unresolved at the moment, it is known that SL signaling leads to an inhibition of both PpCCD7 and PpCCD8 expression (Proust et al., 2011). This negative feedback on SL biosynthesis via corepression of PpCCD7 and PpCCD8 can partly be explained by their close colocalization on chromosome 6. SL production in *P. patens* is moreover induced by phosphate depletion, but it remains to be investigated whether this results from an induction of *PpCCD7* and *PpCCD8* expression at the transcriptional level (Decker et al., 2017).

Like what is hypothesized for jasmonates, there is an ongoing debate that SL might not have been used as hormones *per se* in ancestral plants, but rather interspecific signals, notably permitting symbiosis with symbiotic fungi (Delaux et al., 2012; Waters et al., 2017). On another note, common use of *rac*GR24 in experiments often makes results



unclear, as this enantiomeric mixture not only stimulates the SL signaling pathway but also the euKAI2-MAX2-dependent pathway (Scaffidi et al., 2014), which might also be the case in *P. patens*.

Figure II-3 - Occurrence of plant hormone signaling components in *P. patens***.** Schematic diagrams are shown for each hormone class, with the number of genes found in *P. patens* genome. Receptors are shown as sectors, signal transduction components as ovals and transcription factors as triangles. Components drawn with dotted outlines and question marks indicate still hypothetical involvement in signaling.

CROSSTALKS

Phytohormonal crosstalks involving auxin and/or cytokinins:

The phytohormonal crosstalk that is perhaps the most extensively documented is the interplay of auxin and cytokinins in the induction of gametophore buds. Cytokinin-insensitive bar mutants can produce initial cells but no buds in *P. patens* (Schumaker and Dietrich, 1998). However, addition of auxin can trigger the development of gametophores in these mutants, showing that auxin is also needed for bud assembly, probably at higher concentration than for caulonema differentiation. Conversely, gametophores fail to form in nar auxin insensitive mutants in control conditions. However, when *nar* mutants producing auxin-resistant degron-less PpIAAs are cultured with exogenous cytokinins under low-fluence red light, gametophore formation is partly restored (Prigge et al., 2010). This suggests that cytokinins acts downstream of auxin to promote gametophores formation. Accordingly, BAP treatment results in increased PpIAA gene expression, suggesting that this aspect of the auxin/cytokinins crosstalk, also found in Angiosperms, is ancient. The moss *PpRR10*, a direct target of cytokinin signaling, is repressed both in the degron-less *PpIAA nar* mutants and in WT when treated with NAA. Hence, this suggests that in *P. patens* auxin regulates ARR expression in a PpIAAindependent way (Prigge et al., 2010). On the other hand, Thelander et al. suggested that auxin might sensitize future bud-forming caulonema cells to cytokinins, thereby easing bud initiation (Thelander et al., 2018). Therefore, auxin would act earlier than cytokinins. One notable evidence for that is that the PpAPB1-4 transcription factors that are required for bud initiation are transcriptionally induced by auxin and not by cytokinins (Aoyama et al., 2012). Puzzlingly, auxin and cytokinins then have opposite effects in gametophores branching (Coudert et al., 2015). Auxin is also linked to ethylene, although this was only reported once and not further investigated. In facts, both ACC contents and ethylene formation are promoted by exogenous IAA (Rohwer and Bopp, 1985).

Both cytokinins and CLE peptides are potent regulators of cell division and cell differentiation. In 2019, Cammarata *et al.* described numerous contexts where CLE and cytokinin signaling converge on the same developmental processes in *P. patens*. CLE signaling appears to inhibit the effects of cytokinins at different levels: bud production, regulation of apical meristem identity in gametophores, and cell divisions in phyllids. However, the level(s) at which this inhibition takes place remain(s) to be identified. Still, it is interesting to note that such interplays between cytokinins and CLEs have also been reported in Angiosperms, suggesting these processes are ancestral (Cammarata et al., 2019).

Phytohormonal crosstalks involving stress hormones:

In *Funaria hygrometrica*, cytokinin-mediated bud induction can be inhibited by ABA, in a concentrationdependent manner (Christianson, 2000). More precisely, ABA does not interfere with the initial perception of cytokinin but rather acts by blocking the second step of cytokinins action on bud differentiation, that is the stable commitment to "bud state". Another level of ABA/cytokinins interplay was evidenced later in *P. patens*. Hyoung and colleagues found that the cytokinin inactivation gene *PpCKX1* holds a positive role in resistance to dehydration and salt stress, linked to a higher expression of ABA signaling genes such as *PpABI1A and B* (Hyoung et al., 2020). Thus, cytokinins interact with ABA signaling and likely have an opposite effect on resistance to abiotic stress. ABA signaling is also linked to ethylene signaling, primarily at the level of their shared kinase: PpCTR1L/PpARK. The *Ppctr1l/Ppark* loss of function mutant is completely insensitive to ABA and displays a constitutive response to ethylene, revealing that it plays antagonistic roles in the two pathways (Yasumura et al., 2015). That ancestral dual function for CTR1-like proteins in mediating ABA signaling has likely been lost in the Angiosperms lineage, as it has not been reported in these plants.

In seed plants, ABA and gibberellins have a key antagonistic effect in regulating seed germination. In *P. patens*, exogenously applied *ent*-kaurene similarly reverses the inhibitory effect of ABA on spore germination, however the effects of both hormones are less potent than in seed germination (Vesty et al., 2016). Indeed, all ABA biosynthesis genes are expressed in dry spores and then at lower levels during imbibition, whereas the *ent*-kaurene biosynthesis gene *PpCPS/KS* is expressed only after imbibition, during germination and growth. Likewise, putative ABA signaling genes are for most expressed at higher levels in dry spores than imbibed spores, which is puzzlingly also the case for putative GA/*ent*-kaurene receptors (Vesty et al., 2016).

Crosstalks between light signaling and phytohormones signaling:

A chloronema cell has four possible developmental fates: 1) divide and give rise to new chloronema cells; 2) differentiate into a *caulonema* cell; 3) give rise to a bud; 4) differentiate into brachycytes and *tmema* cells (Decker et al., 2006). Naturally, the energy status has a great impact on the development of the plant in P. patens. This has perhaps been exemplified by the study of the hexokinase mutant *Pphkx1* (Olsson et al., 2003). Glucose produced through photosynthesis is a vital raw material for most of the metabolic pathways and biomass accumulation in plants. However, glucose needs to be phosphorylated via a hexokinase activity before it can enter primary carbon metabolism. PpHXK1 encodes the major, plastid-located, hexokinase in P. patens (Olsson et al., 2003). In WT, glucose treatment induces caulonema formation, showing there is indeed a link between developmental regulation and the energy supply. Thelander and colleagues showed that, as expected, *Pphkx1* has a reduced growth rate and displays a lack of *caulonema* differentiation, even when glucose is provided (Thelander et al., 2005). More surprisingly, cytokinin mediated induction of *chloronema* branching is completely lost in the *Pphxk1* mutant, raising the possibility that cytokinin acts to relieve a *PpHXK1*-dependent inhibition of *chloronema* branching. This is further supported by the fact that *Pphxk1* develop much more buds (even ectopically on primary chloronema) than the cytokinin-treated WT, and that cytokinins do not further increase bud formation in *Pphxk1*. Therefore, cytokinins and hexokinase affect bud formation through a common mechanism, with HXK1 acting downstream (Thelander et al., 2005). Interestingly, the inhibitory effect of auxin on bud development is apparently epistatic to the effect of the *Pphxk1* mutation, which could mean that the auxin-controlled step in bud formation is downstream of the step affected by hexokinase and cytokinins (Thelander et al., 2005). Carbon status is also directly regulated by light and, as discussed therebefore, most phytohormones have been linked to light in a way or another. Partly because of this, response(s) to light is (are) a major crosstalk node involving several hormones in plants. For instance, intact auxin and *ent*-kaurenoic acid signaling are both necessary for red-light induced *caulonema* transition (Hayashi et al., 2010). Indeed, ent-kaurenoic acid deficient mutants have a deficient caulonema differentiation phenotype in red light, rescued by *ent*-kaurenoic acid. In white light also, the *ent*-kaurene deficient *Ppks* mutant is less responsive to NAA than WT and its response is restored when co-treated with *ent*-kaurenoic acid or *ent*-kaurene. Moreover, response to blue light is also disturbed in *ent*-kaurenoic acid deficient mutants: while WT *P. patens* displays an avoidance phenotype to unilateral blue light, both the *Ppks* and the *Ppko* mutants do not, unless exogenous *ent*kaurenoic acid is provided (Miyazaki et al., 2015). *PpKS* gene expression itself is induced by blue light, suggesting GAlike levels might be increased in blue light (Miyazaki et al., 2014, 2015). Thus, *ent*-kaurenoic acid (or active derived compound(s)) in *P. patens* are involved in the well-known crosstalk between light and auxin. Blue light sensed *via* the cryptochrome PpCRY1/b induces side branch formation in the *protonema*, induces leaf growth and represses stem elongation (Imaizumi et al., 2002). Also, since *Ppcry* mutants develop gametophores earlier than the WT, blue light signalled through cryptochromes light likely inhibits the transition to bud, on the opposite of red light. On the other hand, red and blue light both induce *caulonema* formation in WT, and NAA further increases *caulonema* formation under white light, red light, and blue light (Imaizumi et al., 2002). *Ppcry* mutants being more sensitive to NAA except in red light, thus it seems that cryptochrome-mediated blue light signals inhibit auxin responses, whereas red light either induces or does not affect auxin responses (Imaizumi et al., 2002). This inhibition of auxin response is at least partly dependent on IAAs: the *PpIAA1* gene is induced by exogenous auxin in WT and constitutively over-expressed in the double *Ppcry* mutant, which accumulates *PpIAA1* transcripts faster than in the WT under NAA treatment (Imaizumi et al., 2002). Hence, cryptochromes in *P. patens* inhibit auxin signaling by interfering with the induction of auxin responsive genes.

In *P. patens*, cytokinin-induced bud induction requires light, but it has not been determined whether it is initial cell formation or bud assembly (or both) that is light-dependent. Buds cannot develop if the light intensity is too low, even when cytokinins are applied, and this probably depends mainly on the red component of white light. However, rather than light itself, it might be a component only formed in light in some species that is necessary and that might be sucrose, as suggested by Chopra and Gupta for *Funaria hygrometrica* (Chopra and Gupta, 1967). Indeed, it has been shown for a closely related species to *P. patens, Physcomitrium turbinatum*, that there is a relatively large cumulative light dose required for bud formation, suggesting that light energy may be used for the synthesis of a product that must accumulate before buds form. Thus, sugars are the most logical candidates. In *P. patens*, further support for the accumulation of such a product was obtained: some buds are formed when dark-grown cultures are exposed to light for several hours and then simultaneously treated with cytokinin and returned to darkness, suggesting *that P.* patens somehow "remembers" the light signal (and/or cytokinins perpetuate the response to light) (Schumaker and Dietrich, 1998). Interestingly, it was found recently that ABA can be added to the mix, as sucrose inhibits spore germination in a dose-dependent manner and acts synergistically with ABA (Vesty et al., 2016).

CONCLUSION

Appearance of phytohormones groups coincide with (or shortly precede) major transitions in plants evolutionary history and are therefore thought to be particularly relevant for adaptation to new environments and/or new developmental programs. Evolution of biosynthesis and signaling pathways relies on the assembling of pre-existent functions, which is eventually kept by natural selection when it grants a survival asset. These "building blocks", following a long process of co-evolution and possible events of duplication, neofunctionalization and such, finally make up coherent pathways, linking a given signal, synthesized and "freed" in appropriate conditions, to the most appropriate responses.

To study these evolutionary processes, in addition to the fossil records that is often fragmentary or even missing, comparison of plants from diverse lineages is necessary. *Physcomitrium patens* has long been a widely used model species for studies on hormones, thanks to the ease of its *in vitro* propagation and genetic transformation. It has more recently been joined by the liverwort *Marchantia polymorpha*, which also seems to be a promising model for this other bryophyte lineage. We can also note the multiplying of studies on *Selaginella* (Lycophyte), which will undoubtedly help in giving us more insight from this seedless vascular plants' lineage.

Recent reviews gathered much of the current knowledge about the state of phytohormones signaling pathways in various lineages of plants to give us a clearer view of this step-by-step acquisition of signaling components (Bowman et al., 2019; Blázquez et al., 2020).

Chapter III - A short history of strigolactones

III-A) Discovery of the first strigolactones and of their multiple roles in flowering plants

Strigolactones (SL) are a group of apocarotenoids that make up the tenth class of phytohormones, with over 20 molecules identified to date (Xie, 2016; Wang and Bouwmeester, 2018). However, SL were first identified and studied not as phytohormones but as rhizosphere signals. As a matter of facts, strigol was identified in the 1960s as a potent inducer of germination for seeds of the parasitic plant *Striga lutea*, active and produced at hormonal concentrations by its host plant cotton (Cook et al., 1966). The chemical structure of this compound was soon resolved (Cook et al., 1972) and revealed a tricyclic lactone moiety (ABC tricycle) connected to another lactone ring (the methylbutenolide D ring) by an enol-ether bridge. This structure exemplifies the organization of canonical SL, in opposition to non-canonical SL which can bear various groups in place of the ABC moiety (Yoneyama et al., 2018b). However, the enol-ether bridge and D ring are constant features found in all SL (Alder et al., 2012) (figure III-1). Canonical SL can be further divided, depending on the orientation of the C ring, into strigol-type (β orientation) and orobanchol-type (α orientation) (Yoneyama et al., 2018b) and Figure III-1, orobanchol being a germination stimulant for parasitic plants of the *Orobanche* family instead of *Striga* (Müller et al., 1992). It was then demonstrated that SL were derived from carotenoids (Matusova, 2005). The discovery of SL role in parasitism, highly disadvantageous for the producing plant, raised the question as to why SL biosynthesis and exudation into the rhizosphere had not been eliminated by natural selection.

A compound very similar to strigol, 5-deoxystrigol (5DS), was then identified as the sought-after "branching factor" inducing arbuscular mycorrhizal fungi (AMF) hyphal branching (Buee et al., 2000; Akiyama et al., 2005). As such, SL are particularly important for this symbiosis, since hyphal branching is recognized as the host recognition step (Yoneyama et al., 2008). This effect of SL is explained by a rapid increase in AMF mitochondrial division and ATP production, in phylogenetically distant AMF species (Gigaspora rosea, Glomus intraradices and Glomus claroideum) (Besserer et al., 2006). Nevertheless, the mitochondria is not the only cell compartment that is affected by SL in the fungi and SL likely have an even broader effect on fungal gene expression and metabolism (Lanfranco et al., 2017). Moreover, SL also have a positive impact on AMF growth earlier, by promoting AMF spores germination, probably by inducing remobilization of carbon stored in lipids (Besserer et al., 2008; Lanfranco et al., 2017). SL also initiate the molecular dialogue between the host plant and the fungal partner (Genre et al., 2013; Bonfante and Genre, 2015). However, the question as to how SL are perceived by these fungi is still open. A role of SL in the regulation of symbiosis between legumes and *Rhizobia* nitrogen-fixing bacteria has also been investigated (Foo et al., 2014; van Zeijl et al., 2015; De Cuyper et al., 2015; López-Ráez et al., 2017; McAdam et al., 2017), with most studies concluding that SL have an early positive effect on nodulation, by inducing infection thread formation from the bacterial partner end (McAdam et al., 2017). Also, SL biosynthesis in the host plant seems to be induced by its perception of the bacterial Nod factors (McAdam et al., 2017). These roles in symbiosis are highly beneficial to plant growth, and probably outweigh the negative impact of SL-mediated promotion of parasitism in most cases. Hence, the conservation of SL production and exudation along land plants evolution would have been permitted by the evolutive advantage granted by enhanced symbiosis ability.



Figure III-1 – **General view of strigolactones structural diversity.** Opposite stereochemistry of the C ring in orobanchol type SL (blue inset) and strigol type SL (violet inset) is highlighted by blue/violet ovals. Note that the orientation of the D ring (highlighted by red ovals on orobanchol and strigol structures) is always the same in natural SL. The stereochemistry of *ent*5DS and *ent*4DO enantiomers of the artificial SL GR24 is hence said to be non-natural (red inset with dotted outlines). GR24 enantiomers are usually named after the natural SL to which they are the most similar (first notation, similarity is illustrated by the green background). For convenience, GR24 enantiomers will consistently be referred to using the second notation (e.g. (+)-GR24 and so on) in the following chapters.

SL first uncovered hormonal role as repressors of axillary shoot branching was established later, simultaneously by two teams (Gomez-Roldan et al., 2008; Umehara et al., 2008). This finding was permitted by the identification and characterization of hyper-branched mutants in rice (Umehara et al., 2008), pea (Gomez-Roldan et al., 2008) and Arabidopsis (Gomez-Roldan et al., 2008; Umehara et al., 2008). These mutants were subsequently shown to be affected either in SL biosynthesis or signaling. Interestingly, the existence of a plant hormone regulating shoot branching along auxin and cytokinins had been suspected for a long time and previous identification of mutants in genes encoding carotenoid cleavage dioxygenases had shown that this signal was derived from carotenoids (reviewed by Ongaro and Leyser (Ongaro and Leyser, 2008)). Over the years, more developmental roles have been attributed to SL, for some

together with other phytohormones (reviewed in (Brewer et al., 2013; Al-Babili and Bouwmeester, 2015; Lopez-Obando et al., 2015; Yang et al., 2019). Notably, SL induce internode elongation and secondary growth of stems, regulate root architecture depending on the plant phosphate nutritional status, promote senescence in leaves and repress hypocotyl elongation in seedlings. Taken together, these findings suggest that SL generally fine-tune growth and morphogenesis of plants according to the availability of mineral nutrients in the soil, notably inorganic phosphate and nitrogen (Marzec and Melzer, 2018). Furthermore, several other studies point to SL as defense phytohormones against both biotic (Torres-Vera et al., 2014; Decker et al., 2017; Xu et al., 2019; Nasir et al., 2019; Li et al., 2020b) and abiotic stresses (Pandey et al., 2016; Shirani Bidabadi and Sharifi, 2020; Saeed et al., 2017).

III-B) Strigolactones biosynthesis and signaling in flowering plants

SL biosynthesis starts with transformation of all-trans-β-carotene into carlactone (CL, see its structure in figure III-1) in plastids, *via* the successive action of the DWARF 27 (D27) isomerase and of CAROTENOID CLEAVAGE DIOXYGENASE 7 and 8 (CCD7 and CCD8) (Sorefan et al., 2003; Booker et al., 2004; Alder et al., 2012). CL, the precursor of all active natural SL identified so far (Seto et al., 2014), then moves to the cytosol where it is transformed into SL by CYP450 enzymes from the CYP711A/MORE AXILLARY BRANCHING 1 (MAX1) family. This last step(s) are less well known and appear to vary between species, the different MAX1 enzymes likely having very specialized reaction and substrate specificities (Challis et al., 2013; Abe et al., 2014; Zhang et al., 2014; Flematti et al., 2016; Yoneyama et al., 2018a; Iseki et al., 2018; Zhang et al., 2018). Other types of enzymes have also been identified as SL biosynthesis enzymes but have only been found in specific plants. This is notably the case for the 2-oxoglutarate and Fe(II)-dependent dioxygenases LATERAL BRANCHING OXYDOREDUCTASE (LBO) in Arabidopsis (Brewer et al., 2016) and LOTUS-LACTONE DEFECTIVE (LLD) in *Lotus japonicus* (Mori et al., 2020), as well as for LOW GERMINATION STIMULANT 1 (LGS1), a sulfotransferase found in sorghum (Gobena et al., 2017). More recently, another family of CYP450 enzymes, CYP722C, was found to be involved in the generation of canonical SL in cowpea (Wakabayashi et al., 2019) and cotton (Wakabayashi et al., 2020).

SL signaling has been extensively investigated in flowering plants (Arabidopsis, but also pea, petunia and rice). In these species, SL are perceived in the cytosol by DWARF 14 (D14), a peculiar receptor that has a catalytic activity as an α/β -hydrolase (Arite et al., 2009; Yao et al., 2016; de Saint Germain et al., 2016; Yao et al., 2018b). Using this catalytic activity, D14 can cleave its SL ligand, generating a covalently linked D-ring to the histidine residue of the catalytic triad of D14, also called CLIM (for covalently linked intermediate molecule). Moreover, D14 itself adopts a destabilized conformation following its interaction with SL and is eventually degraded by the 26S proteasome (Hu et al., 2017). Before its degradation, the conformational change of D14 enables it to interact in the nucleus with the F-box protein MORE AXILLARY BRANCHING 2 (MAX2) and with proteins of the SUPPRESSOR OF MAX2 1 LIKE (SMAX1-like or SMXL) family (Yao et al., 2016). This multipartite interaction triggers the formation of a SCF^{MAX2} E3 ubiquitin ligase complex that directs SMXL proteins towards proteasomal degradation (Figure III-2). SMXL can thus be seen as repressors of SL responses, which must be removed for the SL signal to be transduced (Jiang et al., 2013; Zhou et al., 2013; Soundappan et al., 2015; Wang et al., 2015b). An ongoing debate about SL signaling is about which state of the D14-SL interaction, either before or after cleavage of the SL and CLIM formation, is the one able to transduce the signal (de Saint Germain et al., 2016; Yao et al., 2016; Carlsson et al., 2018; Marzec and Brewer, 2019). Even though

both states seem able to bind MAX2 and SMXLs, the outcome of these interaction patterns are likely different (Shabek et al., 2018). However, SMXL molecular function(s) are not well understood yet, even more so at the molecular level (more details are given in chapters V, VI and VII). Eventually, SMXL removal has major effects on gene expression (Wang et al., 2020b), notably lifting the transcriptional block on *BRC1* (Braun et al., 2012), a TCP transcription factor acting as a major repressor of axillary branching. Nonetheless, some effects of SL signaling are transcription independent, such as the relocation of PINs auxin efflux transporters, which additionally does not require *de novo* protein synthesis (Shinohara et al., 2013; Kumar et al., 2015). This rapid effect also has a negative effect on branching, since it prevents exportation of auxin out of axillary buds, blocking them in a dormant state. Both transcription-dependent and transcription-independent processes play together in SL response, probably at variable levels depending on the mechanism. Notably, it seems that SL effect on branching relies mainly on the former, whereas SL regulation of stem secondary growth is more dependent on the latter (Liang et al., 2016).



Figure III-2 – Schematic overview of SL biosynthesis and signaling pathways. SL responses include repression of axillary branching and regulation of many other developmental processes detailed in part III-B.

III-C) The mystery of the doppelgänger KL pathway

Simultaneously to the progressive elucidation of the SL signaling pathway, evidence for another pathway having multiple levels of convergence with the former emerged. In this second pathway, the same MAX2 F-box protein and close homologs of D14 (KAI2, also an α/β -hydrolase) and SMXL proteins (SMAX1/SMXL2) are respectively acting as receptor and negative regulator (Scaffidi et al., 2013; Waters et al., 2012b). On another note, SMAX1 has been identified in Arabidopsis in a suppressor screen in *max2* (Stanga et al., 2013), the same year as the SL pathway SMXL D53 in rice (Jiang et al., 2013; Zhou et al., 2013). However, the *smax1* mutation only suppresses the *max2* phenotypes that are not associated with SL signaling, that is germination and seedling photomorphogenesis (Stanga et al., 2013). The KAI2-MAX2-SMAX1 pathway enables response to karrikins (KAR), exogenous butenolides found in smoke generated from burning vegetation (figure III-3). In fire-following species, these compounds trigger rapid seed germination, even when other positive environmental signals are lacking (especially light). In regards to that, it is interesting to note that the *KAI2 (KARRIKIN INSENSITIVE 2*) gene had first been characterized as *HTL (HYPOSENSITIVE TO LIGHT*) (Sun and Ni, 2011).



Figure III-3 – Structure of compounds perceived through the KAI2-MAX2 pathway

The f-box protein MAX2 itself had been independently identified as KAI1 (KARRIKIN INSENSITIVE 1) and its dual role in SL and KAR pathways has been shown in Arabidopsis already 10 years ago (Nelson et al., 2011). Interestingly, MAX2 had been identified in earlier screens as a gene regulating senescence (as ORE9) (Woo et al., 2001) and photomorphogenesis (as PPS) (Shen et al., 2007). Recognition that kai2 and max2 loss-of-function mutants have major developmental defects, together with the observation that these genes exist and have similar developmental functions in plants that are not fire-following, or even insensitive to KAR, pointed to this pathway likely recognizing an endogenous molecule (Flematti et al., 2013; Conn and Nelson, 2016). Still, this endogenous KAI2 ligand (KL) has not been identified yet, even though several studies sought to clarify its nature (Scaffidi et al., 2013; Conn and Nelson, 2016). Some divergences between the two pathways exist: for instance, enzymatic activity of KAI2 does not seem to be necessary for KL signaling (Yao et al., 2018a; Zheng et al., 2020). But they work similarly in the way that SMAX1 is degraded in a MAX2-dependent manner after its interaction with KAR-activated KAI2 (Khosla et al., 2020; Zheng et al., 2020). Extensive use of an unspecific racemic mixture of the synthetic SL analogue GR24 (racGR24), as well as studies relying only on the max2 loss-of-function mutants, have made attribution of diverse physiological functions to either SL or KL signaling challenging (De Cuyper et al., 2017). Studies interested in the evolution of these twin pathways seem to converge on the KL pathway (KAI2-MAX2-SMAX1, figure III-4) being ancestral relative to the SL pathway (D14-MAX2-SMXL, Figure III-2) (Bythell-Douglas et al., 2017; Walker et al., 2019). This particular point on evolution is further discussed hereafter in chapters V and VIII. Another ongoing investigation is how MAX2 can differentiate between SL and KL signals to target different SMXL and whether it relies only on which receptor (KAI2 or D14) is activated at a given time (given possible co-expression of the D14, KAI2, MAX2, SMAX1 and SMXL7 genes in some tissues, according to the Arabidopsis eFP Browser expression atlas). Also, if them both being activated at the same time is possible, does MAX2 « prioritize » one signal over the other?



Figure III-4 Schematic overview of the putative KL signaling pathway

III-D) Strigolactones biosynthesis and signaling outside of flowering plants

All extant land plants explored so far, including those that are hosts neither for parasitic plants nor for AMF, possess the basic genetic toolkit for SL biosynthesis until carlactone production (thus until CCD8 function) (Delaux et al., 2012; Walker et al., 2019). Moreover, the loss of MAX1 and LBO close homologs seems specific to *P. patens* lineage and is not even a common feature of mosses, which could imply that most bryophytes are able to synthesize SL downstream of carlactone using enzymes of the MAX1 and LBO families (Walker et al., 2019). This conservation of "CCD8-derived compounds" biosynthesis also infers that such non-host plant species either use these compounds in other types of interspecific communications, or that these compounds are "restrained" to their phytohormonal functions. This second hypothesis is especially supported by the current view that canonical SL act mainly in the rhizosphere as interspecific signals, whereas non-canonical SL are the actual phytohormones (Yoneyama et al., 2018b). Answering this question is further complicated by the extreme difficulty to identify SL molecules, as they are produced in very low amounts and furthermore highly unstable in aqueous solutions. Broadened to ancestral land plants, these interrogations are aiming to determine what was the first role of SL: an interspecific signal acting in the environment or an endogenous growth-regulating signal?

According to a recent study (Decker et al., 2017), the moss *P. patens* can produce several molecules from PpCCD7 and PpCCD8 activity. When expressed *in vitro* and fed with all *trans*- β -carotene, PpCCD7 is indeed able to synthesize the 9-cis-beta-apo-10'-carotenal SL precursor from 9-*cis*- β -carotene. But, PpCCD7 is also able to cleave other types of carotenoids: it can convert both 9-*cis*-zeaxanthin and 9'-*cis*-cryptoxanthin into 9-*cis*-3-OH- β -apo-10'-

carotenal, 9-*cis*-lutein into 9-*cis*-3-OH- α -apo-10'-carotenal, and 9-*cis*-cryptoxanthin into 9-*cis*-3- β -apo-10'-carotenal. However, the biological relevance of these other apocarotenoids, also produced by CCD7 in several angiosperms, remains to be investigated. *In vitro*, PpCCD8 can produce carlactone from 9-cis-beta-apo-10'-carotenal, thus it is a functional carlactone synthase. PpCCD8 can also transform all-*trans*- β -apo-10'-carotenal into β -apo-13-carotenone (better known as d'orenone). Whether these molecules are all produced *in planta* is unknown, in addition to their function in most plant species, especially *P. patens*. Nevertheless, findings presented in chapter IV do suggest that *P. patens* synthesizes non-canonical SL from PpCCD7 and PpCCD8 conjoined action, putatively after the isomerization of all-*trans*- β -carotene by a PpD27-like (PpD27L) homolog. *P. patens* possesses five *PpD27L* genes (table III-1). Following the consideration that genes involved in the same pathway are usually co-expressed, comparison of expression profiles suggests that PpD27L2 is most probably acting upstream of PpCCD7 and PpCCD8. Indeed, these three genes are collectively most highly expressed in rhizoids whereas their transcript levels are very low in sporophytes and spores (figure III-5). However, if we consider that the most promising candidate is the one subjected to negative feedback by PpCCD8-derived compounds, as *D27* homologs from Angiosperms (Waters et al., 2012a) then *PpD27L1* might be the best candidate (our unpublished data).

Genes homologous to aforementioned constituents of the SL signaling toolkit are found in all land plants examined so far (Knack et al., 2015; Wang et al., 2015a; Walker et al., 2019) (table III-1). Still, some of these homologs are very distant, so them having a similar function to their characterized angiosperms' counterparts is far from being obvious. This is particularly the case for the putative SL receptors, and this issue is further complexified by the existence of a highly similar pathway in response to another putative class of phytohormones. Globally, the mechanisms underlying SL perception and signal transduction outside of flowering plants are still very unclear. The results presented herein in chapters IV and VI refine current knowledge about these processes in the model moss species *P. patens*, as well as signaling associated with the elusive KL.



Figure III-5 – **Expression profile of putative SL biosynthesis genes in** *Physcomitrium patens* Absolute levels of expression are given according to the data from Ortiz-Ramirez available on *P. patens* eFP Browser (Ortiz-Ramírez et al., 2016) (http://bar.utoronto.ca/efp_physcomitrella/cgi-bin/efpWeb.cgi). Genes ID are respectively *Pp3c9_19810 (PpD27L1), Pp3c26_11090 (PpD27L2), Pp3c4_23850 (PpD27L3), Pp3c24_12440 (PpD27L4), Pp3c4_23900 (PpD27L5), Pp3c6_21550 (PpCCD7)* and *Pp3c6_21520 (PpCCD8).*

Gene function	A. thaliana	P. sativum	O. sativa	P. patens
β-carotene isomerase	AtD27		D27	5 <i>PpD27L</i>
CCD7	MAX3	RMS5	D17/HTD1	PpCCD7
CCD8	MAX4	RMS1	<i>D10</i> + 2 <i>D10L</i>	PpCCD8
Cytochrome P450	MAX1	2 PsMAX1	CO, OS, etc.	
(CyP711A1)				
			(5 OsMAX1)	
2-oxoglutarate and Fe(II)-	LBO	PsLBO	OsLBO	
dependent dioxygenase				
Receptor	AtD14	RMS3	D14	13 PpKAI2L
F-box protein	MAX2	RMS4	D3	<i>PpMAX2</i> (?)
ClpATPase	<i>SMXL6</i> ,7 et 8	PsSMXL6	D53 and D53L	<i>PpSMXL A, B, C</i> and <i>D</i>
		(<i>DOR3</i>),7 et 8		
TPL transcriptional	4 TPL	Several TPL	3 TPL	2 TPL
corepressor				
TCP transcription factor	AtBRC1	PsBRC1	OsTB1	8 TCP

 Table III-1 – Homologs of SL related genes in flowering plants and the moss Physcomitrium patens.

RESULTS AND DISCUSSION

CHAPTER IV – *Physcomitrium patens* receptors to strigolactones and related compounds highlight MAX2 dependent and independent pathways.

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Authors contributions:

S.B, A.dSG, M.L-O and C.R designed the project. M.L-O, A.G, F-D.B, D.C, B.H, P.L.B, J-B.P conducted the experiments: A.dSG carried out biochemistry experiments with help from A.G for some experiments. S.B and A.G conducted experiments on Arabidopsis. Experiments on Phelipanche ramosa were done by J-B.P. Most of the molecular biology work was done by P.L.B. A.G, M.L-O, F-D.B, D.C, P.L.B, J-B.P, P.D, C.R, A.dSG and S.B analyzed the data. A.G and S.B wrote the manuscript, with essential contributions from M.L-O, F-D.B, P.D, C.R and A.dSG.

Short title

Defining receptors to strigolactones in a moss

Abstract

In flowering plants, strigolactones (SL) are perceived by an alpha/beta hydrolase, DWARF14 (D14), that interacts with the F-box protein MORE AXILLARY GROWTH2 (MAX2) for regulating developmental processes. The key SL biosynthetic enzyme, CAROTENOID CLEAVAGE DEOXYGENASE8 (CCD8), is present in the moss

Physcomitrium (Physcomitrella) patens, and PpCCD8-derived compounds regulate plant extension. However, their exact nature and receptors remain unknown. Germination assays with two populations of the parasitic plant, *Phelipanche ramosa,* indicate that PpCCD8-derived compounds could be non-canonical SLs. We previously reported that the PpMAX2 homolog is not involved in PpCCD8-derived compounds perception. 13 *PpKAI2LIKE-A to -L* genes, homologous to the ancestral *D14* paralog *KARRIKIN INSENSITIVE2 (KAI2)* are found in the moss genome. In Arabidopsis, AtKAI2 is the receptor for karrikins and a still elusive endogenous KAI2-Ligand (KL). Here, we show that all tested PpKAI2L proteins can bind and cleave SL analogs, some with similar affinities as AtKAI2. Strikingly, the PpKAI2L-H protein shows a strong hydrolytic activity not found in the other PpKAI2L. Moss mutants for all *PpKAI2L* genes encode redundant proteins that are not involved in PpCCD8-derived compounds perception, but rather act in a PpMAX2-dependant pathway. In contrast, mutations in *PpKAI2L-G*, and *-J* genes abolish the response to the SL analog (+)-GR24, making both encoded proteins the best candidate receptors for the PpCCD8-derived molecules.

Introduction

Strigolactones (SLs) are butenolide compounds with dual roles in plants: exuded in soil, SLs signal the presence of a host to Arbuscular Mycorrhizal (AM) fungi (Akiyama et al., 2005; Besserer et al., 2006), and thus favor the establishment of symbiosis; as endogenous compounds, SLs (or derived compounds) play a hormonal role in developmental program (Gomez-Roldan et al., 2008; Umehara et al., 2008; for review: Lopez-Obando et al., 2015; Waters et al., 2017). SLs exuded from plant roots can also act in the rhizosphere as a signal molecule inducing the germination of seeds of parasitic plants (Cook et al., 1966; Delavault et al., 2017). SLs have been found in most land plants, including Bryophytes, Lycophytes, Gymnosperms, and Angiosperms (Yoneyama et al., 2018b). However, their synthesis and signaling pathways are mainly described in the latter, where a core pathway of enzymes among which two CAROTENOID CLEAVAGE DEOXYGENASE (CCD7 and CCD8) convert carotenoids into carlactone (CL), the reported precursor of all known SLs. CL is the substrate for further enzymes as the CYTOCHROME-P450 MORE AXILLARY GROWTH1 (MAX1) (for review: Alder et al., 2012; Al-Babili and Bouwmeester, 2015). Depending on plant species, CL is modified into canonical or non-canonical SLs, that differ in the structure attached to the conserved enol ether-D ring moiety, shared by all the SLs, and essential for biological activity (Yoneyama et al., 2018b; Yoneyama, 2020). In Angiosperms, SLs are perceived by an α/β hydrolase DWARF14 (D14)/DECREASED APICAL DOMINANCE2 (DAD2) that, in interaction with an F-box protein MORE AXILLARY GROWTH2 (MAX2), targets repressor proteins for proteasome degradation (Soundappan et al., 2015; Wang et al., 2015b). SLs perception originality is that the D14 protein is both a receptor and an enzyme that cleaves its substrate (and covalently binds part of the SL), in a mechanism that is still debated (Yao et al., 2016; de Saint Germain et al., 2016; Shabek et al., 2018; Seto et al., 2019; for review: Bürger and Chory, 2020).

The evolutive origins of SLs, and in particular the identification of their primary role, as hormones or rhizospheric signals are still elusive. SLs identification and quantification are challenging in many species, due to very low amounts of the molecules present in plants and its exudates, and their high structure diversification (Xie, 2016; Yoneyama et al., 2018b). Therefore, occurrence of SLs in a species often relies on the presence of the core biosynthesis enzymes in its genome (Delaux et al., 2012; Walker et al., 2019). Besides, it has been recently proposed that SLs are only produced in land plants (Walker et al., 2019). As for perception, evidence of an ancestral pathway came from the

finding during the screening of Arabidopsis mutants of an ancient paralog of D14, named KARRIKIN INSENSTIVE2/HYPOSENSITIVE TO LIGHT (KAI2/HTL) (Nelson et al., 2011; Waters et al., 2012b). KAI2 is an α/β hydrolase as D14, that also interacts with the MAX2 F-box protein, in a pathway regulating Arabidopsis seed germination and seedling development. However, the endogenous signal perceived through this pathway remains unknown and is reported thus far as KAI2-Ligand (KL) (Conn and Nelson, 2016). KAI2 is also involved in stress tolerance, drought tolerance and AM symbiosis (Gutjahr et al., 2015; Wang et al., 2018; Villaécija-Aguilar et al., 2019; Li et al., 2020).

To gain insight into SLs signaling evolution, we focused our investigations on a model for earliest land plants, namely Physcomitrium (Physcomitrella) patens (P. patens). As a moss, P. patens belongs to bryophytes, which also include two other clades, hornworts and liverworts (Bowman et al., 2019). Bryophytes are currently assigned as a monophyletic group of embryophytes sharing an ancestor with vascular plants (Puttick et al., 2018; Harris et al., 2020). Extant bryophytes are therefore considered as the descendants of the first plants which became able to survive out of water, and conquer land, 450 million years ago (Bowman et al., 2019; Blázquez et al., 2020; Harris et al., 2020). In P. patens, both CAROTENOID CLEAVAGE DEOXYGENASE7 and 8 (PpCCD7 and PpCCD8) enzymes required for SL synthesis are found (Proust et al., 2011), and carlactone has been detected as the product of PpCCD8 (Decker et al., 2017). The extended phenotype of the *Ppccd8* mutant plants indicates that PpCCD8-derived molecules are required for moss filament growth regulation, and more broadly for interaction with neighboring plants (Proust et al., 2011). Application of the artificial SL (±)-GR24 does complement the *Ppccd8* mutant phenotype (Proust et al., 2011). PpCCD8derived molecules likely play also a role in rhizoid elongation and gametophore shoot branching (Delaux et al., 2012; Coudert et al., 2015). The exact nature of PpCCD8-derived molecules is still elusive (Yoneyama et al., 2018b) and the absence of MAX1 homologs in *P. patens* suggests that the synthesis pathway in moss may differ from that in vascular plants. However, phylogenetic analysis of MAX1 homologs highlights the presence of this gene in other mosses and suggest a conservation of the biosynthesis pathway in land plants (Walker et al., 2019). Contrary to its flowering plants homolog, we previously showed that PpMAX2 is not involved in the perception of PpCCD8-derived molecules, as the corresponding mutant does respond to (±)-GR24 (Lopez-Obando et al., 2018). The PpMAX2 F-box protein is rather involved in a light dependent pathway required for moss early development and gametophore development regulation (number and size) (Lopez-Obando et al., 2018). In a first attempt no true homolog for the D14 SL receptor was found in P. patens genome, whereas 11-13 PpKAI2-LIKE (PpKAI2L) candidate genes, named PpKAI2L-A to PpKAI2L-M, were described (Lopez-Obando et al., 2016a). These genes are split in 4 subclades (i), (i.i-i.ii), (ii) and (iii), hereafter renamed respectively as PpKAI2L-(A-E), (F,K), (H,I,L), and (J,G,M). A comprehensive phylogenetic assessment described the presence of moss clades (F,K), (H,I,L), and (J,G,M)) into a super clade called DDK (D14/DLK2/KAI2) containing spermatophyte (Angiosperm and Gymnosperm) D14 clades, while clade (A-E) belongs to the EuKAI2 clade, common to all land plants (Bythell-Douglas et al., 2017). Nevertheless, the characteristics of moss proteins from DDK clade were found to be as different from D14 as from KAI2 (Bythell-Douglas et al., 2017). Structure prediction of the PpKAI2L proteins indicated various pocket sizes, as observed for D14 and KAI2 from vascular plants (Lopez-Obando et al, 2016). Consequently, those (or some of those) genes could encode receptors for various molecules, including the PpCCD8-derived compounds, or the elusive KL (Conn and Nelson, 2016). Accordingly, our work on PpMAX2 allowed us to hypothesize that this F-box protein might be involved in a moss KL pathway (Lopez-Obando et al., 2018). But the question as to the involvement of PpKAI2L proteins in the PpMAX2 pathway remains open. Last year, Bürger et al.

published the crystal structure of PpKAI2L-C, -E and -H and showed that *in vitro* purified PpKAI2L proteins -C, -D and -E bind (-)-5-deoxystrigol, a canonical SL with non-natural stereochemistry, while PpKAI2L proteins -H, -K and -L could adapt the karrikin KAR₁ (Bürger et al., 2019). However, proteins from the (J,G,M) clade were not studied, and no evidence for a role of one (or several) PpKAI2L as receptor for CCD8-derived molecules was brought, nor *in vivo* (in moss) experiments validating the results.

Several questions remain to understand the evolution of the SL signaling pathway, and we address the following two in this paper: what is the nature of PpCCD8-derived molecules in moss? What are the receptors for these compounds? Answering these questions would be facilitated if one could mimic these molecules in assays. So far, the racemic (±)-GR24 has been used as an analog to SLs, but recent works indicate that the different enantiomers present in this synthetic mixture do not have the same effect (Scaffidi et al., 2014). Indeed, (+)-GR24 (also called GR24^{5DS}), with a configuration close to natural strigol is mostly perceived by D14 and mimics CCD8-derived SLs (e.g. carlactone), while (-)-GR24 (also called GR24^{ent5DS}) has a configuration that so far has not been found in natural SLs. However, the (-)-GR24 analog is better perceived by KAI2 than by D14 proteins and has been described as a KL mimic (Scaffidi et al., 2014; Zheng et al., 2020).

Here we tested the activity of moss as a stimulant for *P. ramosa* germination to shed light on the chemical nature of PpCCD8-derived compounds. We characterized the expression patterns of all 13 *PpKAI2L* genes in moss, at various developmental stages. Refining the findings of Bürger *et al.* (2019), we assessed the biochemical activity of PpKAI2L proteins *in vitro* by testing their cleavage activity and binding towards both GR24 enantiomers. We expressed some of these proteins in Arabidopsis *d14-1 kai2-2* double mutant to question the conservation of the SL and/or KL perception function. Finally, we used CRISPR-Cas9 technology to isolate moss mutants amongst the four clades of *PpKAI2L* genes and analyzed their phenotype and their response to both GR24 enantiomers. We propose that clade (A-E) PpKAI2L proteins could be moss KL receptors, while clade (J,G,M) PpKAI2L would function as PpCCD8-derived compounds receptors.



Figure IV-1 - PpCCD8-derived compounds are germination stimulant (GS) of a specific group of *Phelipanche ramosa*. (A) GS activities of exudates of *P. patens* on seeds of *P. ramosa* group 1 and 2a relative to (\pm) -GR24 0,1 μ M. (B) Germination rate of seeds of *P. ramosa* group 2a on plates with *P. patens* WT, *Ppccd8* mutant plants, or with culture medium only, with or without (\pm) -GR24 0.1 μ M. (C) Seeds from *P. ramosa* group 1 (left) and group 2a (right) on plates with WT (top) or *Ppccd8* mutant (bottom), with or without (\pm) -GR24 0,1 μ M. Arrows point at germinating seeds. Scale bar = 0.5 mm.

Results

PpCCD8-derived compounds induce the germination of a hemp-specific population of Phelipanche ramosa

Although Yoneyama et al. (2018) noted that the PpCCD8-derived molecules, previously reported in moss tissues by Proust et al. (2011), were likely contaminants, it is still a valuable hypothesis that P. patens does synthesize SL like products. Indeed, the synthetic SL analog (\pm)-GR24 or carlactone do complement the *Ppccd8* phenotype (Proust et al., 2011; Decker et al., 2017). Unfortunately, quantification of SL and related compounds is still a challenge in many species (Boutet-Mercey et al., 2018; Yoneyama et al., 2018b; Rial et al., 2019; Floková et al., 2020). An alternative to evaluate the SL-like activity of PpCCD8-derived molecules is to test the effect of moss exudates as stimulant of parasitic seeds germination. Parasitic plants as *Phelipanche ramosa* can parasitize various host plants, in response to specific exuded germination stimulants (GS). Indeed, different genetic groups of P. ramosa seeds can be the identified, depending on the crop grown in the field where seeds have been collected (Huet et al., 2020). Seeds from two populations of *P. ramosa* harvested in hemp (*P. ramosa* group 2a) and oilseed rape (*P. ramosa* group 1) fields (Stojanova et al., 2019; Huet et al., 2020) were assayed towards WT moss exudates. As control, both groups of seeds germinate in presence of (±)-GR24 (Figure IV-1A). Germination of *P. ramosa* group 2a seeds, but not *P. ramosa* group 1, is induced by WT moss exudates (Figure IV-1A). In another assay, seeds from the P. ramosa 2a population were added in culture plates close to WT or *Ppccd8* plants, with and without (±)-GR24 (Figure IV-1B-C). *P. ramosa* group 2a seeds germinate on WT moss plates, while no germination is observed in the vicinity of *Ppccd8* plants. In both cases (WT and *Ppccd8*), the addition of (±)-GR24 to the medium restores seed germination. Thus, PpCCD8-derived compounds induce the germination of a specific population of *P. ramosa* seeds, responding to GS exuded by hemp.



Figure IV-2 - Phenotypic response to (+)- and (-)-GR24 enantiomers and natural compounds: number of *caulonema* filaments. *Caulonema* filaments were counted in WT (A) and *Ppccd8* SL synthesis mutant (B) grown 10 days vertically in the dark, following application of increasing concentrations (0.1, 1 and 10 μ M) of (+)-GR24 (cyan boxes), (-)-GR24 (red boxes) and KAR₂, (blue boxes). Control is 0.01% DMSO. (C) *Caulonema* filament numbers of WT and *Ppccd8* mutant grown 10 days vertically in the dark, following application of increasing concentrations of increasing concentrations (0.1, 1 and 10 μ M) of (±)-Z-CL (noted Z-CL, green boxes). Control is 0.01% DMSO. (+)-GR24 (cyan boxes) and (-)-GR24 (red boxes) were applied at 1 μ M. Significant differences between control and treated plants within a genotype based

on a Kruskal-Wallis test, followed by a Dunn *post-hoc* test for multiple comparisons: ***, P<0.001; **, P<0.01; *, P<0.5; For each genotype and treatment, n = 24 plants grown in 3 different 24 well-plates.

P. patens strongly responds to (+)-GR24 and carlactone application, but poorly to (-)-GR24 and KAR2 in the dark

So far, the length of *caulonema* filaments grown in the dark was used as a proxy to quantify the *P. patens* phenotypic response to SL using (±)-GR24 in Petri dishes assays (Hoffmann et al., 2014; Lopez-Obando et al., 2018). With 24-well tissues culture plates, we reduced the amount of compound required for phenotypic assays (Guillory and Bonhomme, methods chapter given in annex 1) and the number of caulonemal filaments per plant ended up as a more robust proxy of the response to compounds than *caulonema* length (Figure IV-2 and Supplemental Figure IV-1). Since it is now well established that the (-)-GR24 can activate nonspecific responses (Scaffidi et al., 2014), we tested separately the (+) and (-)-GR24 enantiomers on both WT and *Ppccd8* mutant. As in previous studies (Hoffmann et al., 2014; Lopez-Obando et al., 2018), we predict a clearer response in the synthesis mutant than in the WT, due to absence of endogenous PpCCD8-derived compounds potentially mimicked by (+)-GR24. Both the number and the length of caulonema filaments significantly decrease following application of (+)-GR24 in WT and Ppccd8 mutant, in a doseresponse manner (Figure IV-2 and Supplemental Figure IV-1). A dose of 0.1 µM is enough to see a clear and significant response in terms of number in both genotypes (Figure IV-2). Application of (-)-GR24 leads to a significant decrease of Ppccd8 filaments length, as (+)-GR24, but has no effect on WT filament length (Supplemental Figure IV-1). Strikingly, no significant changes of *caulonema* filaments number is observed with (-)-GR24, except in WT for which 0.1 µM and 10 µM doses leads to a significant increase of this number (more pronounced at 0.1 µM, Figure IV-2A). Higher concentrations of (-)-GR24 are not significantly active in WT, nor in *Ppccd8*. However, in further assays (see below, Figure IV-11), a slight but significant decrease of *Ppccd8 caulonema* filament number is observed following (-)-GR24 application. To summarize, the phenotypic response of *P. patens* is significant and marked with the (+)-GR24 that induces a decrease of both filament number and length in WT and *Ppccd8*. The response to (-)-GR24 is less clear for both genotypes, with sometimes contrary effects (increase of number in WT, see above).

A previous study concluded on the absence of response to KAR₁ in both WT and *Ppccd8* mutant, when testing the *caulonema* length in the dark or using a transcriptional marker (Hoffmann et al., 2014). In the present work, we tested the KAR₂ molecule described as more active than KAR₁ in Arabidopsis (Sun et al., 2020) (Figure IV-2A-B). KAR₂ has an unmethylated butenolide group, unlike KAR₁. In WT, no significant effect on *caulonema* number is observed following application of increasing doses of KAR₂ (Figure IV-2A). In *Ppccd8*, we observe an increase of filament number, as with (-)-GR24 in WT, and this increase is significant at 10 μ M (Figure IV-2B). Surprisingly, the length of WT filaments is significantly decreased by the 1 μ M dose only, while higher concentration (10 μ M) has no significant effect (Supplemental figure IV-1). *Caulonema* length is also diminished in the *Ppccd8* mutant by KAR₂ application but at low concentrations (0.1 μ M and 1 μ M, Supplemental Figure IV-1). To conclude, KAR₂ phenotypic effects on *P. patens* are slight and not clearly dose responsive, similarly to those of (-)-GR24.

We also tested racemic Z-carlactone (CL), described as the natural product of PpCCD8 in *P. patens* (Decker et al., 2017). CL application has a negative effect on *caulonema* filament number of both WT and *Ppccd8* mutant, however significant only at 10 µM (Figure IV-2C).

With these phenotypic assays, we can conclude to separate effects of GR24 enantiomers in *P. patens*, as in Arabidopsis (Scaffidi et al., 2014). Indeed, the (+)-GR24 analog mimics CL effects, though it is far more potent, and can thus be used to mimic the PpCCD8 compounds effects, while the (-)-GR24 analog has slight phenotypic effects, that resemble those of KAR₂ molecule.





used are V3.1 (downloaded from the Phytozome website in September 2019) for all *PpKAI2L* genes except for *PpKAI2L-B*, *PpKAI2L-H* and *PpKAI2L-M* (V3.2). Regions targeted by guide RNAs are signaled by black inverted triangles, with their names written in bold italic. Light blue, orange and light green bands respectively represent codons for the S, D and H residues of the catalytic triad (see Supplemental Table 3 for reference sequences).

All *PpKAI2L* genes are expressed at relatively low levels and putatively encode proteins with a conserved catalytic triad

Figure IV-3 shows the predicted phylogenetic tree (Figure 3A) and the structure (Figure 3B) of all 13 *PpKAI2L* genes, along with that of *AtD14* and *AtKAI2*. The catalytic triad (Ser, His, Asp) is found in all genes (Supplemental Figure 2, alignment), including *PpKAI2L-A* and *M* for which recent sequencing data (Physcomitrella patens v3.3, Lang et al, 2018) contradicts previous pseudogenes predictions. In the following, the four subclades (previously described in Lopez-Obando et al, 2016), will be renamed for convenience: clade (A-E) for clade (i) including PpKAI2L-A, -B, -C, -D, and -E, clade (F,K) for clade (ii) including PpKAI2L-F and -K, clade (H,I,L) for clade (i.i-i.ii) including PpKAI2L-H, -I, -L, and clade (J,G,M) for clade (iii) including PpKAI2L-J, -G, and -M.

We obtained the expression pattern of all *PpKAI2L* genes in *P. patens*, thanks to a cDNA library from various organs/tissues (Supplemental Figure 3 A), including spores, *protonema* of increasing age and different tissue composition (6-day-old: primarily *chloronema*; 11-day-old: mix of *chloronema* and *caulonema*; 15-day-old: mix of *chloronema*, *caulonema* and gametophores buds), and gametophores from 5-week-old plants. *PpKAI2L* genes transcripts are found in all tested tissues, at relatively low levels compared to the control genes. Notably, we also find that *PpKAI2L-A* is expressed, thus confirming it is not a pseudogene. This could not be assessed by qPCR for *PpKAI2L-M*, as its predicted transcript is almost identical to *PpKAI2L-G*, and reported transcript levels are attributed to both *PpKAI2L-G* and *-M*. Thus *PpKAI2L-G/M* probably arose recently as a local duplication in the genome of *P. patens*. In spores, *PpKAI2L-D* from clade (A-E) shows the highest transcript levels compared to any other *PpKAI2L* genes. *PpKAI2L-I* transcript levels are the lowest, in all tested tissues. When considering the clades separately (Supplemental Figure 3B-E), *PpKAI2L-D* has higher transcript levels among clade (A-E) genes, while *PpKAI2L-H* transcript levels are highest compared to that of *PpKAI2L-I* and *-L* (clade (H,I,L)). *PpKAI2L-G/M* (clade (F,K)) show comparable transcript levels, and *PpKAI2L-J* has slightly higher transcript levels than *PfKAI2L-G/M* (clade (J,G,M)). The data are consistent with those previously reported (Ortiz-Ramirez et al, 2016, shown in Supplemental Figure IV-4).



Figure IV-4 - PpKAI2L proteins response differential to the GR24 isomers. (A) Chemical structures of GR24 isomers. (B-J) Thermostability of AtD14, AtKAI2 and PpKAI2 proteins at 10 μ M in absence of ligand (black line) or in presence of various ligands (+)-GR24 (blue line), (-)-GR24 (red line), (+)-2'*-epi*-GR24 (green line) and (-)-2'*-epi*-GR24 (purple line) at 100 μ M analyzed by nanoDSF. For each proteins the top panels show the changes in fluorescence (ratio F_{350nm}/F_{330nm}) with temperature, whereas the bottom panels show the first derivatives for the F_{350nm}/F_{330nm} curve against the temperature gradient from which the apparent melting temperatures (T_m) for each sample was determined. The experiment was carried out twice.



Figure IV-4 - PpKAI2L proteins response differential to the GR24 isomers. Continued.

PpKAI2L-C, -D, -E proteins are destabilized by (-)-GR24 as AtKAI2, while PpKAI2L-F, -K, -L and -H weakly interact with GR24 enantiomers

To investigate whether the PpKAI2L proteins behave similarly to AtD14 or AtKAI2 *in vitro*, seven PpKAI2L (-C,-D,-E,-F,-H,-K and -L) protein CDS were cloned for over-expression in *E. coli*, and successfully purified, in order to test their stability, interaction with SL analogs and potential enzymatic activities. Unfortunately, because of low solubility, not any of the six other PpKAI2L proteins, especially none of the clade (J,G,M) PpKAI2L could be purified in sufficient amount to perform quality controls as for the others.

To test the interactions of PpKAI2L purified proteins with SL analogs, we used both nanoDSF (Figure IV-4) and classical DSF (Supplemental Figure IV-5). AtD14 is destabilized by all four GR24 isomers, with the (+)-2'-*epi* enantiomer being the least potent inducer of destabilization (Figure IV-4B and Supplemental Figure IV-5B). AtKAI2 Tm decreases following (-)-GR24 addition, indicating that it is destabilized by (-)-GR24 (Figure IV-4C and Supplemental Figure IV-5A), as previously reported (Waters et al, 2015). All tested clade (A-E) proteins (PpKAI2L-C -D and -E) are destabilized by (-)-GR24 addition, as AtKAI2 (Figure IV-4D-F and Supplemental Figure IV-5C-E). Puzzingly, PpKAI2L-C, -D and -E proteins show a tendency to be stabilized by (+)-GR24 at high concentration (Supplemental Figure IV-5C-E). The other PpKAI2L proteins all show different DSF profiles. Only PpKAI2L-K (clade (F,K)) is destabilized by both (+)-GR24 and (-)-GR24, and stabilized by (+)-2'-*epi*-GR24 (Tm + 1.6 °C) (Figure IV-4I). PpKAI2L-L (clade (H,I,L)) shows a slight increase of the Tm following addition of all four isomers ($\leq 1^{\circ}$ C), suggesting a slight stabilization (Figure IV-4J), that has not been observed in a previous study (Bürger et al., 2019). The stability of the PpKAI2L-F (clade (F,K)) and PpKAI2L-H (clade (H,I,L)) proteins is not affected by any of the four isomers (Figure IV-4G-H and Supplemental Figure IV-5F-G).

The binding affinity of the PpKAI2L proteins for the GR24 isomers was further quantified by K_d affinity calculations following intrinsic tryptophan fluorescence measurements (Figure IV-5 and Supplemental figure IV-6). Both PpKAI2L-D and PpKAI2L-E show a comparable affinity for (-)-GR24, (respectively 92 μ M and 39 μ M), similar to that recorded for AtKAI2 (45 μ M) and for AtD14 (94 μ M) (Figure IV-5A-D). K_d value for (+)-GR24 couldn't be determined due to low affinity of these two proteins. While no change of the PpKAI2L-H protein stability is observed in nanoDSF, interaction between this protein and all three GR24 stereoisomer ((+)-GR24, (-)-GR24 and (-)-2'-*epi*-GR24) is detected (Figure IV-5G). K_d values between 100 μ M and 200 μ M are estimated, indicating a weak affinity for these compounds. Finally, intrinsic fluorescence assays with PpKAI2L-F and PpKAI2L-K confirm a behavior that is different between the two proteins from clade (F-K), as well as from proteins of the other clades: PpKAI2L-F doesn't seems to interact with any GR24 stereoisomers, while PpKAI2L-K shows an affinity of 41 μ M towards (-)-GR24 and 107 μ M towards (+)-GR24 (Figure IV-5E-F).

To summarize, all proteins from clade (A-E) have a similar biochemical behavior toward SL analogs, with a stereoselectivity toward (-)-GR24, similar to AtKAI2. As to the proteins from the other tested clades, (H,I,L) and (F,K), no specific feature can be highlighted, indicating that these proteins need to be considered independently regarding their interaction with the ligand. Furthermore, these results show that (+)-GR24 and (-)-GR24 stereoisomer can be bound by PpKAI2L proteins from different clades, and even lead to opposite effects, in terms of stability.



Figure IV-5 - SL isomers bind PpKAI2L proteins based on intrinsic tryptophan fluorescence with different affinity. Plots of fluorescence intensity *versus* SL concentrations. The change in intrinsic fluorescence of AtD14 (A);

AtKAI2 (B); PpKAI2L-D (C); PpKAI2L-E; (D) PpKAI2L-F (E); PpKAI2L-K (F) and PpKAI2L-H (G) was monitored (see Supplementary Figure IV-4) and used to determine the apparent K_d values. The plots represent the mean of two replicates and the experiments were repeated at least three times. The analysis was performed with GraphPad Prism 8.0 Software.

PpKAI2L-C, -D, -E, and -K preferentially cleave the (-)-GR24, while PpKAI2L-H and -L cleave all four GR24 enantiomers

As most PpKAI2L proteins were able to bind at least one of the GR24 enantiomers, and since the catalytic triad is conserved in all of them, the next step was to test their enzymatic activity against SL analogs. When incubated with the generic substrate for esterases, 4-nitrophenyl acetate (p-NPA), all tested PpKAI2L proteins show enzymatic activities, as well as AtKAI2 (Supplemental Figure IV-7A-B). Kinetic constants are in the same range for all proteins, similar to AtKAI2, to the exception of PpKAI2L-H, which shows higher V_{max} and K_M , highlighting a faster catalysis and a better affinity for *p*-NPA than all the others (Supplemental Figure IV-7C). The PpKAI2L proteins enzymatic activity was then tested towards the four GR24 isomers, and compared to that of pea SL receptor, RMS3/PsD14, and AtKAI2 (Figure IV-6A). All three PpKAI2L-C -D and -E (clade (A-E)) show comparable enzymatic stereoselectivity towards GR24 isomers (between 15 and 20%), close, though significantly lower, to that of AtKAI2 that reached 30%. In contrast to AtKAI2, PpKAI2L-C -D and -E can also cleave the other isomers, nevertheless at low level (5-10%) suggesting a less stringent selectivity. As for PpKAI2L proteins from clade (F,K), PpKAI2L-F shows very low enzymatic activity towards all four isomers (less than 5%), while PpKAI2L-K enzymatic activity is comparable to that of clade (A-E) and to that of PpKAI2L-L (clade (H,I,L)). Finally, although none of the PpKAI2L proteins shows as high catalytic activity as RMS3 (100% cleavage activity towards (+)-GR24, (-)-GR24 and (-)-2'-epi-GR24), the PpKAI2L-H protein shows a significant high catalytic activity towards all four GR24 isomers, especially towards (-)-GR24 (almost 70%, Figure IV-6A).


Figure IV-6 - PpKAI2L enzymatic activities confirm stereoselectivity and reveal PpKAI2L-H special feature. (A) PpKAI2L enzymatic activity towards GR24 isomers. (+)-GR24, (-)-GR24, (+)-2'-*epi*-GR24 and (-)-2'-*epi*-GR24 at 10 μ M were incubated with RMS3, AtKAI2 and 7 PpKAI2 proteins at 5 μ M for 150 min at 25 °C. UPLC-UV (260 nm) analysis was used to detect the remaining amount of GR24 isomers. Columns represent the mean value of the hydrolysis rate calculated from the remaining GR24 isomers, quantified in comparison with (±)-1-indanol as internal standard. Error bars represent the SD of three replicates (means ± SD, n = 3). The asterisks indicate statistical significance from the AtKAI2 values, for each isomer, as ***p ≤ 0.001; **p ≤ 0.01 and n.s., p > 0.05, as measured by Dunnett nonparametric relative contrast effects test, with AtKAI2 taken as the control group, for each compound. (B) Enzymatic kinetics for PpKAI2L, AtD14 and AtKAI2 proteins incubated with (±)-GC242 (structure shown Supplemental Figure IV-8A). Progress curves during probe hydrolysis, monitored (λ_{em} 460 nm) at 25 °C. Protein catalyzed hydrolysis with 330 nM of protein and 4 μ M of probe. These traces represent one of the three replicates and the experiments were

repeated at two times. (C) Sequence alignment of active site amino acid residues for PpKAI2 proteins. Amino acids that differ from AtKAI2 are colored in red. A fully expanded alignment can be found in Supplemental Figure IV-2. (D) Superimposition of the AtD14 and PpKAI2L-H structure showing the position of the F²⁸ and L²⁸ residues. Zoom onto helices α 4 and α 5. (E) Enzymatic kinetics for PpKAI2L-H, PpKAI2L-H^{L28F} and AtD14 proteins incubated with (±)-GC242. Progress curves during probe hydrolysis, monitored (λ_{em} 460 nm) at 25 °C. Protein catalyzed hydrolysis with 330 nM of protein and 20 µM of probe. These traces represent one of the three replicates and the experiments were repeated at two times. (F) Hyperbolic plot of pre-steady state kinetics reaction velocity with (±)-GC242. Initial velocity was determined with pro-fluorescent probe concentrations from 0.310 µM to 40 µM and protein at 400 nM. Error bars represent SE of the mean of three replicates and the experiments were repeated at least three times.

The high hydrolysis activity of the PpKAI2L-H protein and the lack of thermal shift when incubated with GR24 isomers suggests a different behavior of this protein compared to other PpKAI2L. To better characterize this enzymatic activity, we used as substrate a pro-fluorescent probe ((\pm) -GC242), where the ABC rings of GR24 are replaced by a coumarin-derived moiety (DiFMU) (de Saint Germain et al., 2016). (±)-GC242 is bioactive on moss, as the number of caulonema filaments in the dark is reduced by this compound, in a dose response manner (evaluated on *Ppccd8* mutant, Supplemental figure IV-8A). The use of (\pm) -GC242 as substrate confirms PpKAI2L-H high enzymatic activity, versus all other PpKAI2L proteins (Figure IV-6B). Indeed, after 2 hours, PpKAI2L-H catalyzes the formation of 1µM DiFMU, while other PpKAI2L activities are not distinguishable from background noise. However, PpKAI2L-H enzymatic profile doesn't show a biphasic curve (a short burst phase, quickly followed by a plateau phase), that characterizes the AtD14 single turnover activity (Figure IV-6B, de Saint Germain et al., 2016). The lack of plateau for PpKAI2L-H rather suggests that this protein acts as a Michaelian enzyme with SL analogs. To try to understand this singularity, we compared the solvent exposed residues in the binding pocket of the PpKAI2L proteins and noticed that PpKAI2L-H harbors a Leucine²⁸ residue instead of Phenylalanine (Figure IV-6C) found in AtD14 (F²⁶), AtKAI2 and all other PpKAI2L proteins. The F residue is located at the junction between helix $\alpha 4$ and $\alpha 5$, nearby the catalytic site, and can precisely interact with the D-ring of the SL (Figure IV-6D). Furthermore, a mutant PpKAI2L-H protein where L²⁸ is changed into F shows a biphasic cleavage profile similar to AtD14, both reaching a plateau at 0.4µM DiFMU, corresponding to the protein concentration (Figure IV-6E-F). PpKAI2L-H and PpKAI2L-H^{L28F} proteins have comparable affinity towards (±)-GC242 ($K_{1/2}$ = 4,794 µM vs 4,675 µM) but show different Vmax values (V_{max} =0,06794 μ M.min⁻¹ vs 0,01465 μ M.min⁻¹), suggesting that the L²⁸ residue affects the velocity of catalytic activity. Thus, the PpKAI2L-H protein lacks a Phenylalanine residue that partly explains its strong enzymatic activity.



Figure IV-6 - PpKAI2L enzymatic activities confirm stereoselectivity and reveal PpKAI2L-H special feature. Continued.

Moss PpKAI2L proteins covalently link GR24 enantiomers, as vascular plants receptors

To further question the role of PpKAI2L proteins as receptors, we looked for covalent attachment of the GR24 isomers to the PpKAI2L proteins. (Supplemental Figure IV-9). Mass spectrometry analyses highlight 96 Da increments (corresponding to the D ring mass), when incubating AtKAI2 and (-)-GR24, and all PpKAI2L-C, -D, -E, -F or -L with (-)-GR24. Strikingly, 96 Da increments are also observed when incubating PpKAI2L-E -F -L and -K with the other isomer (+)-GR24. However, for PpKAI2L-E, the peak intensity is much lower with (+)-GR24 than with (-)-GR24, confirming the better affinity for the latter (Figure IV-5). PpKAI2L-H does not covalently bind the D ring, following incubation with either of both enantiomers, further arguing for a Michaelian enzymatic activity and a specific enzymatic role for this protein. Poor interactions are observed with (+)-GR24, reported as mimicking SLs, and showing the more potent effect in our phenotypic assays (Figure IV-2 and Supplemental Figure IV-1). Strikingly, all clade (A-E) PpKAI2L proteins show strongest affinity for the (-)-GR24, reported in vascular plants as a mimic for KL unknown compound (Scaffidi et al., 2014; Zheng et al., 2020).

Biochemical analysis of PpKAI2L proteins thus highlights some resemblances with the AtD14 and even more with the AtKAI2 protein, in accordance with a possible role as SL (or related compounds) and/or KL receptors. *In planta* studies were next led to further test this possible role.

None of the *PpKAI2L* gene complements the Arabidopsis *d14-1 kai2-2* double mutant

We used cross species complementation assays to test if some of the PpKAI2L proteins could ensure similar function to that of AtD14 and/or AtKAI2 in Arabidopsis SL and/or KL signaling. *PpKAI2L* (C, D, F, G, J, H) genes were cloned downstream the AtD14 or AtKAI2 promoters, and resulting constructs were expressed in the Arabidopsis double mutant At*d14-1 kai2-2*, that shows both hyperbranched phenotype and elongated hypocotyls (Supplemental Figure IV-10A). As controls, the double mutant was transformed with AtKAI2 or AtD14 CDS under the control of endogenous promoters. Only lines expressing AtD14 under the control of the AtD14 promoter fully restored the rosette branching to WT (Ler) values (Figure IV-7A). Under the control of AtD14 promoter, neither AtKAI2 nor any of the PpKAI2L can significantly restore the branching phenotype of the At*d14-1 kai2-2* mutant, to the exception of one line expressing PpKAI2L-J (#4.6), that shows a significant lower number of rosette branches (still higher to that of WT and AtD14 expressing line, Figure IV-7A). We conclude that none of the *PpKAI2L* genes can fully complement the AtD14 function in shoot branching.

We tested possible complementation of AtKAI2 function in the At*d14-1 kai2-2* mutant, by monitoring hypocotyl length under low light conditions, with or without 1 μ M (+)-GR24 or (-)-GR24 in the culture medium (Figure IV-7B). Compared to WT, the double mutant shows longer hypocotyls in control conditions, as the single *kai2-2* mutant, and neither (+)-GR24 nor (-)-GR24 addition has an effect on this phenotype. In contrast, 1 μ M (+)-GR24 in the medium leads to shorter *kai2-2* hypocotyls, likely due to perception and transduction by the AtD14 protein still active in the single mutant. Accordingly, 1 μ M (-)-GR24 in the medium has no effect on *kai2-2* hypocotyls. Expressing AtKAI2 under the control of AtKAI2 promoter surprisingly does not fully restore the hypocotyl length of the double mutant in our control conditions, but it does restore the response to (-)-GR24 as expected. similar function to AtD14 in a *kai2-2* background, for hypocotyl development but not for rosette branching.

More surprising, PromAtKAI2:AtD14 expressing line shows longer hypocotyls than the double mutant in control conditions (DMSO), and similar phenotypes of longer hypocotyls in control conditions were found in lines expressing PpKAI2L-C (Supplemental Figure IV-10), PpKAI2L-J and PpKAI2L-H (Figure IV-7B). This unexpected effect of the introduced α/β hydrolases will be discussed below. In contrast, only lines expressing PpKAI2L-G had short hypocotyls in control conditions, suggesting a possible restoration of AtKAI2 function by the expressed protein. When either (+)-GR24 or (-)-GR24 is added, short hypocotyls (similar to WT) are observed in the PromAtKAI2:AtD14 expressing line, indicating AtD14-mediated signal transduction of both enantiomers. However, adding GR24 enantiomers in the medium had no such effect on lines expressing PpKAI2L-H that showed a clear response to (+)-GR24 addition (shorter hypocotyls). To conclude with these assays, PpKAI2L-G is able to mediate the Arabidopsis KL signaling in hypocotyls, though it does not fully ensure AtKAI2 response function; PpKAI2L-H is able to ensure similar function to AtD14 in a *kai2-2* background, for hypocotyl development but not for rosette branching.



Figure IV-7 - Complementation assays of Arabidopsis Atd14-1 *kai2-2 double* **mutant with** *PpKA12L* **genes.** Complementation assays of Atd14-1 *kai2-2* mutant (in Ler), transformed using the *AtD14* promoter (A) or *AtKA12* promoter (B) to control AtD14, *AtKA12* (controls) or *PpKA12L* genes as noted below the graph. Ler (WT), *kai2-2* and Atd14-1 *kai2-2* mutants are shown as controls. (A) Number of rosette axillary branches per plant. Results are mean of

n =12 plants per genotype, except for Ler and lines PromAtD14:AtD14 #12 and PromAtD14::PpKAI2L-C#24.3 (n = 11). Different letters indicate significantly different results between genotypes based on a Kruskal-Wallis test (P < 0.05, Dunn *post hoc* test with P values corrected following the Benjamini-Hochberg method). (B) Hypocotyl length under low light, on ½ MS medium with DMSO (control, grey bars) 1 μ M (+)-GR24 (blue bars) or 1 μ M (-)-GR24 (red bars). Different letters indicate significantly different results between genotypes in control conditions based on a Kruskal-Wallis test (P < 0.05, Dunn *post hoc* test with P values corrected following the Benjamini-Hochberg method). Symbols in blue and red give the statistical significance of response to (+)-GR24 and (-)-GR24 respectively (Mann-Whitney tests, * 0.01 ≤ p < 0.05, *** p ≤ 0.001).

Multiplex editing of all PpKAI2L genes

Multiplex Gene Editing using CRISPR-Cas9 allows to knock down several genes in a single transformation experiment (Lopez-Obando et al., 2016b). Guide RNAs were chosen in each *PpKA12L* gene, preferably in the first exon, to ideally obtain the earliest nonsense mutation possible. When no guide could be designed in the first exon, it was alternatively chosen to recognize a region in close proximity to the codon for one of the last two residues of the catalytic triad (Figure IV-1B). The obtained mutations in each gene are described on Figure IV-8 and Supplemental Figure IV-11. Following the DNA repair, small deletion (1-75 bp) and/or insertion-deletion events led to aa deletions, or frame shift in the predicted protein sequence. We noted with an * the predicted knock out mutations due to premature STOP codons or large deletion induced in the mutant sequences. The use of five guide RNAs simultaneously (Supplemental Table 1) allowed to isolate mutants affected in all of clade (A-E) genes, as well as other mutants affected in fewer genes of this clade (see Lopez-Obando et al., 2016b and Supplemental table IV-2). We chose two triple (*Ppkai2L-a2*-b4** $c2^*$, $Ppkai2L-c2^*-d4^*-e1$) and two quintuple mutants ($Ppkai2L-a1-b1-c1-d1-e2^*$ and $Ppkai2L-a3^*-b1-c3^*-d3^*-e2^*$) for further analysis (Supplemental Table IV-2). The three other clades (H,I,L), (F,K), and (J,G,M) were targeted in separate experiments using combinations of specific guide-RNAs for each gene (*PpKAI2L-F* to -L). The same guide-RNA was used to target *PpKAI2L-G* and *-M*. Several mutants were obtained for clade (J,G,M) genes, including a single KO Ppkai2L-i mutant (i1*) (Supplemental Table IV-2). As biochemistry experiments suggested a pure enzymatic role for PpKAI2L-H, a deletion mutant was obtained through homologous recombination, where the full CDS was removed from the moss genome (Δh mutant, Figure IV-8 and Supplemental Figure IV-12A). This Δh mutant was chosen for further transformation experiments with guide-RNAs from the same (H,I,L) clade and/or from clade (F,K) and (J,G,M), leading to Δh -*i*-*l* and Δh -*f*-*k*-*j* mutants (Supplemental Table IV-2). Eventually, a 7X mutant was obtained (Δh -*i*-*f*-*k*-*j*-*g*m) where all but mutations in PpKA12L-J and -M genes were null mutations. (Supplemental Table IV-2 and see hereafter).

	Mutation		Effect
PpKAI2L-A	01100	21 - β2 41	
WT GAGATTGTTGTCCTCGGCCATGGTTT <u>TGG</u> CACCGACCAGTCTGTGTGGA		EIVVLGHĞFGTDQSVWKHVVP	
a1 GAGATTGTTGTCCTCGGCCATGGTACCGACCAGTCTGTGTGGA	- 6bp	EIVVLGHGTDQSVWKHVVP	- 2 AA
a2* GAGATTGTTGTCCTCGGCCATGACGTTT <u>TGG</u> CACCGACCAGTCTGTGTGGA	+ 2pb	EIVVLGHDVLAPTSL-/49/*	frame shift
23* GAGATTGTGTGTCCTCGGCCATG-GACCAGTCTGTGTGGA	quii -	EIVVLGHGPVCVEICKISPR*	irame shiit
PpKAI2L-B		1 19	
WT ATGTGTCCGGGACCGACTCTGT <u>TGG</u> AGGCTCATAAT		MCPGPTLLEAHNVHVVGHG	
b1 ATGTGTCCGGGACCGACTCATAAT	-12bp	MCPGPTHNVHVVGHG	- 4
b4* ATGTGTCCGGGACCGACATGCTTCCATAC-TGT <u>TGG</u> AGGCTCA TAA T	-13bp;+12bp	MCPGPTCFHTVGGS*	frame shift
PpKA12L-C		53 ————————————————————————————————————	
WT GATAACATGGGAGCTGGCA <u>CCA</u> CCGACCCGGAGTATTTTAGCTTCTCGAGA		DNMGAGTTDPEYFSFSRYST	
c1 GATAACCCGGAGTATTTTAGCTTCTCGAGA	-21bp	DNPEYFSFSRYST	- 7 AA
c2* GATAACATGGGAGCTGGCACCACCGGAGTATTT TAG CTTCTCGAGA	-5bp	DNMGAGTTGVF*	frame shift
C3* GATAACATGGGAGCTGGCA <u>CCA</u> CCGAAGTATTT TAG CTTCTCGAGA	qac-	DNMGAGTTEVF*	irame sniit
PpKAI2L-D		23 β2 42	
WT GGTCATGGGTTCGGCACGGATCAATCTGTGTGGAAGCACGTCATTCCACAC		VVLGHGFGTDQSVWKHVIPH	
d1 GGTCATGGGTTCGGCACGGATCGTGTGTGGAAGCACGTCATTCCACAC	-4bp;+1bp	VVLGHGFGTDR-VWKHVIPH	-1 AA & Q33H
d3* GGTCATGGAAGCACGTCATTCCACAC	-25bp	VVLGHGSTSFHTLSMSTV*	frame shift
a4* GUTCATGGGTTCGGCACGGATCAATCCACAC	-200p	VVLGHGFGTDQSTPCK*	Irame shii
PpKAI2L-E		90 <u>β4</u> αC 108	
WT ATCTACGTTGGACATTCGGTGGCCGGTATGGTTGGTTGTCTCGCATCGCT TGA		SCIÝVGHSV-AGMVGCLASL	
e1 ATCTACGTTGGACATTCGGTGGACATTCGTATGGTTGGTTGTCTCGCATCGCTTGA	-3bp;+6bp	SCIYVGHSVDIRMVGCLASL	+1AA & AG99IH
e2* ATCTACGTTGGACATTCGTATGGTTGGTTGTCTCGCATCGCT TGA	qa8–	SCIYVGHSYGWLSRIA*	frame shift
PpKA12L-F		17 38	
WT GGGTTTGGATCTGACCAGACGGTGTGGAAATACGTCTTGCCGTAT		VLSHGFGSDQTVWKYVLPYIMN	
f1* GGGTTACGTCTTGCCGTAT f2* GGGTACGTCTTGCCGTAT	-19pb -19pb	VLSHGFGNTSCRIS* VLSHGCGNTSCRIS*	frame shift
	10 500		
WT CCGCTCCAAGTGGCGAATTACATGGTACGGAACTTGGGAGGGTGGACGATGA k1* CCGCTCCAAGTGGCGAATTACATGGTACGGAACTTGGGAGGGTGGACGATGA	+7bp	PLQVANIMVKNLGGWIMM	frame shift
k2* CCGCTCCAAGTGGCGAATTACATGTACGGAACTTGGGAGGGTGGACGA TGA	-1bp	PLOVANYMYGTWEGGR*	frame shif
	-	~	
WT CGCTTTTTCTTTCCAACTGCCTCGAAGAGATGCCGAGCCCGTTGC-//-ACTGCATCGA	CTCATGATCTA	TCCAAAACAGCCAT	CDS deletion
Ah CGCTTTTTCTTTCCAACTGCCTCGAAGAGCTCGAGATAACTTC-/(LoxP)/-GAACTT	ATACTAGTCTA	TCCAAAACAGCCAT	
Ррка121-1 МТ СТСТТССАТАТАТАТСССАСССССССССССССССССС	5	LEDIMGAGTTDPEHESSK	
i1* CTGTTCGATATAATGGGAGCCGGCACTTTAGCTCTAAGAGT	-16bp	LFDIMGAGTLALR-/13/-RF*	frame shift
<i>i2*</i> CTGTTCGATATAATGGGAGCCCGGAGCACTT TAG CTCTAAGAGT	-14bp	LFDIMGAGAL*	frame shift
i3* CTGTTCGATATAATGGGAGCCCGGCACCCTGAGCACTTTAGCTCTAAGAGT	-7bp	LFDIMGAGTLSTL-/16/-RF*	frame shift
PpKAI2L-L		54 <u>β1</u> 68	
WT TTGTTAGCTGCCCACAATCTGAGTGTTCTGGGCCAACGGCGACCAAGTT	11bp	LLAAHNLSVLGNGDQV	fromo chift
12 TTGTTAGCTGCCCATCTGAGTGTTCTGGGGCAACGGCGACCAAGTT	-3bp	LLSECSGORRPSC/0/ ~	
<u> </u>			
		Not	
		14 conserved 30	
	-6bp:+5bp	DLCHENPYNVEGSO/20/*	frame shift
j3 GACTTGTGCCACCTGAAGGCTCACAACGTAAGTGTCTTC	-12bp	DLCHLKAHNVSVF	-4 AA
j4 GACTTGTGCCACGAGAACCCTTACCACGTAAGTGTCTTC	-12bp	DLCHENPYNVSVF	-4 AA
<i>j5</i> GACTTGTGCCACGAGAACCCTTACTACGTAAGTGTCTTC	-13bp;+1bp	DLCHENPYYVSVF	-4aa & N26Y
PpKAI2L-G	:	157 <u>β6</u> αT1 175	
WT CAGTAACGACGGAGATTACATCGGAGGGTTTGAGATGGAGGAGCTTCATGAGCTGTT	126-	SNDGDYIGGFEMEELHELF	4 70 70
gi CAGIAACGACGAAGATTACAT-CGGAGGGGTTTGAGATGGAGGAGCTTCATGAGCTGTT a2* CAGTAACGACGGAGATTACAT	-120p -28bp	SNDGDYMSCSPPCCOIS*	-4AA frame shift
al castal c	-64bp	SNDGDYI	-21AA
g^{4*} CAGTAACGACGGAGATGGAGGAGTTT GA GATGGAGGAGCTTCATGAGCTGTT	-11bp;+6bp	SNDGDGGV*	frame shift
PpKAI2L-M		157 <u>β6</u> αT1 175	
WT AGTAACGACGAGAGTTACATCGGAGGGGTTTGAGATGGAGGAGCTTCATGAGCTGTTC	1.01	SNDGDYIGGFEMEELHELF	43.3
mi AGTAAUGACGGAGGGTTTTGAGATGGAGGAGCTTCATGAGCTGTTC	-12b	SNDGFEMEELHELF	-4AA frame shift
m2 AGTAACGACGAGATTACAT-GGAGGGGITTGAGAGGAGGAGGAGGAGGTTCATGAGCTGTTC m6* AGTAACGACGGAGATTACATCGGTTTTGAGATGGAGGAGGAGGTCTCATGAGCTGTTC	-10p -4bp	SNDGDYIGLRW/12/15*	frame shift
	12p		

Figure IV-8 - Mutations obtained in all 13 *PpKAI2L* genes. For all 13 *PpKAI2-Like* genes, WT nucleotide and protein sequences are shown, above altered sequences found in various CRISPR-Cas9 lines (in italics, numbered). The number

of first shown amino-acid (aa) and the predicted secondary structure are indicated above the WT protein sequence. The sgRNA sequence is shown in blue, with the PAM site underlined. Deletions are shown as dashes, insertions are noted with orange letters. The mutation type is shown on the right. Premature STOP codons are noted in bold, and with a red star on the aa sequence. On protein sequences, the number of not shown aa is noted between slashes. For *PpKAI2L-E*, the serine (S) of the catalytic triad is noted in bold blue. For *PpKAI2L-H*, a deletion of the full coding sequence between ATG and STOP was obtained through homologous recombination; the use of CRE recombination led to 46 residual nucleotides, (not shown) corresponding to the LoxP site (see Methods). See Supplemental Table IV-2 for the list of mutants carrying one or several of the shown mutations.



Figure IV-9 - *Ppkai2L* mutant phenotype in light. Plant diameters were measured after 4 weeks growth in the light. (A) 3-week-old plants. Scale bar = 2 mm (B) and (C): 30-day-old plants, n > 40; (D): 28-day-old plants, n = 30. (B), (C) and (D): All plants were grown on cellophane disks. Letters indicate statistical significance of comparisons between all genotypes (Kruskal-Wallis test followed by a Dunn *post hoc* test (p < 0.05)).

The *Ppkai2L* clade (A-E) quintuple mutants phenocopy *Ppmax2-1* in white light, while mutants in other clades are more similar to WT or *Ppccd8*

Our rationale was that a mutant affected in the response to PpCCD8-derived compounds should show a phenotype similar to that of the *Ppccd8* synthesis mutant. We first performed a phenotypic analysis of the mutants in light conditions. After 4 weeks of culture, the *Ppccd8* mutant plant size is slightly larger than that of the WT (Proust et al., 2011, Figure IV-9), while the *Ppmax2-1* mutant is smaller, with fewer but bigger gametophores (Lopez-Obando et al., 2018, Figure IV-9). The diameter of mutants in clade (A-E) genes is significantly smaller than that of *Ppccd8* and WT, and a bit larger than that of *Ppmax2-1* (Figure IV-9A-B and Supplemental Figure IV-12B). The phenotype of the clade (A-E) mutants, with early and big gametophores resembles that of *Ppmax2-1* mutant despite not as strong (Figure IV-9A). To the naked eye, 3 week-old plants from mutants of all three clades (F,K) (J,G,M) and (H,I,L) genes are indistinguishable from WT (Figure IV-9A). These observations also stand for very young plants (10-day-old, Supplemental Figure IV-12B). After a month growth however, all mutants affecting genes from clade (F,K) and (J,G,M) show slightly larger diameter, intermediate between that of WT and *Ppccd8*, the triple *Ppkai2L j3-g3*-m1* being even larger than *Ppccd8* (Figure IV-9C and D). Mutants in clade (H,I,L) as *Ppkai2L \Dh. Ppkai2L \Dh.i2** and *Ppkai2L \Dh.i2**.



Figure IV-10 - *Ppkai2L* mutant gametophores in red light. (A) Gametophore height of *Ppkai2L* mutants affecting clade (A-E) genes ($a2^*-b4^*-c2^*$; $c2^*-d4^*-e1$; $a1-b1-c1-d1-e2^*$) and clade (H,I,L) genes ($\Delta h-i2^*$ and $\Delta h-i3^*-l1$), compared to that of WT, *Ppccd8* and *Ppmax2-1* mutants, following 2 months growth under red light. Mutant genotypes carry mutations as indicated in Figure IV-8 and Supplemental Table IV-2, with asterisks for null mutations. Box plots of n = 32-36 gametophores, grown in 3 Magenta pots, harboring between 15 and 25 leaves. Statistical groups (all genotypes comparison) are indicated by letters and were determined with a Kruskal-Wallis test followed by a Dunn *post hoc* test (p<0.05). (B) Gametophore height of *Ppkai2L* mutants affecting clade (H,I,L) gene (Δh), both clades (F,K) and (J,G,M) ($f2^*-j5^*-k2^*$) and all 3 clades (H,I,L) (F,K) and (J,G,M) ($\Delta h-f1^*-j4-k1^*$ and $\Delta h-f1^*-j4-k1^*-g2^*-m1$). Mutant genotypes comparison) are indicated in Figure IV-8 and Supplemental Table IV-2, with asterisks for null mutations. Box plots of n = 11-15 gametophores, grown in 3 Magenta pots, harboring between 15 and 25 leaves. Statistical groups (all genotypes comparison) are indicated by letters and were determined with a Kruskal-Wallis test followed by a Dunn *post hoc* test (p < 0.05). (C) Examples of gametophores following 2 months growth under red light, from WT, *Ppccd8*, *Ppmax2-1*, and *Ppkai2L* mutants as shown in (A) and (B). Scale bar = 5 mm.

Clade (A-E) quintuple mutants are affected in photomorphogenesis, as *Ppmax2*

Mutants in clade (A-E) genes show the typical phenotype of the *Ppmax2-1* mutant in white light. We previously showed that *Ppmax2-1* mutant is affected in photomorphogenesis under red light (Lopez-Obando et al., 2018). Following 2 months growth under red light, *Ppmax2-1* gametophores are much more elongated than WT gametophores, whereas *Ppccd8* mutant gametophores are shorter (Figure IV-10). Among mutants affecting clade (A-E) genes, both triple mutants *Ppkai2L a2*-b4*-c2** and *Ppkai2L c2*-d4*-e1* gametophores show similar height to WT. Interestingly, the quintuple mutant (*Ppkai2L a1-b1-c1-d1-e2**) shows significantly elongated gametophores, similar to *Ppmax2-1* (Figure 10 A and C). The other tested quintuple mutants (*Ppkai2L a3*-b1-c3*-d3*-e2** and *a1-b1-c1-d1-e2**) also show elongated gametophores under red light, intermediate between WT and *Ppmax2-1* (Supplemental Figure IV-13). The weak phenotype of both triple mutants suggests a functional redundancy among clade (A-E) genes, as KO mutations for PpKAI2L-A, B, C and/or D do not lead to as elongated gametophores as in *Ppmax2-1* mutant.

Gametophores from mutants where genes from clade (F,K) and/or (J,G,M) were mutated (*Ppkai2L f2*-j5-k2**, *Ppkai2L \Delta h-f1*-j4-k1** and Δh -f1*-j4-k1*-g2*-m1, Figure 10B) are similar in height to WT, suggesting that genes from clade (F,K) and clade (J,G,M) have no role in photomorphogenesis in red light. The *Ppkai2L \Delta h-i2** and *Ppkai2L \Delta h-i3*-l1* mutants, affected in clade (H,I,L) genes show shorter gametophores under red light, similar to *Ppccd8* (Figure IV-10A, C), while the single *Ppkai2L \Delta h* mutant shows gametophores intermediate in height between WT and *Ppccd8* (Figure IV-10B). This may suggest a specific role for the (H,I,L) clade genes, opposite to that of *PpMAX2*, or unrelated to the PpMAX2 pathway.

In conclusion, the phenotype of the *Ppkai2L* mutants in red light allows to separate clade (A-E) genes, likely involved in a PpMAX2 dependent pathway, related to photomorphogenesis, and genes from the three other clades, likely independent from this pathway.



Figure IV-11 - Phenotypic response of *Ppkai2L* mutants to (+)-GR24 and (-)-GR24 application in the dark. (A)(B) *Caulonema* numbers from mutants affecting clade (A-E) genes following application of 0.1 μ M (-)-GR24 (in red, A) or 0.1 μ M (+)-GR24 (in cyan, B). DMSO was applied as control treatment (ctl, dark grey). WT and both *Ppccd8* and *Ppmax2-1* mutants were used as control genotypes. (C-E) *Caulonema* numbers from mutants affecting clade

(J,G,M) genes: $j1^*$; j3- $g3^*$ -m1; $j1^*$ - $g2^*$ - $m2^*$; clade (F,K) and (J,G,M) genes: $f2^*$ - $k2^*$ -j5, clade (H,I,L) genes: Δh , Δh - $i2^*$, Δh - $i3^*$ - $l1^*$, clade (H,I,L) and (J,G,M) genes: $j1^*$ -g1- $i3^*$ -l2, or all 3 clades genes: Δh - $f1^*$ -j4- $k1^*$, following application of 0.1µM (-)-GR24 (in red, C) or 0.1µM (+)-GR24 (in cyan, D, E). 0.01% DMSO was applied as control treatment (ctl, dark grey). WT and *Ppccd8* mutant were used as control genotypes. Mutant genotypes carry mutations as indicated in Figure IV-8 and Supplemental Table IV-2, with asterisks for null mutations. For each genotype, *caulonema* were counted after 2 weeks in the dark, from 24 individuals, grown in 3 different 24-well plates. Statistical groups (comparing genotypes in control conditions) are indicated by letters and were determined by a one-way ANOVA with Welch test (95% Cl). Significant differences between control and treated plants within a genotype based on one-way ANOVA with Welch test: ***, p < 0.001; **, p < 0.01; *, p < 0.5; , p < 0.1.

Mutants in (J,G,M) clade do not respond to (+)-GR24 application

We report above the response of WT and *Ppccd8* mutant to SLs analogs (Figure IV-2). To determine among the *Ppkai2L* mutants those carrying mutations in potential receptors for PpCCD8-compounds (SL-related) or other (KL-related) compounds, we tested their phenotypic response in the dark to GR24 enantiomer application, using 0.1 μ M concentration in all assays, and counting the number of *caulonema* filaments per plant.

For the (A-E) clade, in control conditions, all *Ppkai2L* mutants show a number of filaments equivalent to that of WT, except for the quintuple mutant, which tends to have less filaments in control conditions, like *Ppmax2-1* (Figure IV-11A-B and Supplemental Figure IV-14A). No significant effect of (-)-GR24 0.1 μ M addition is observed on clade (A-E) mutants, as for WT (Figure IV-11A). In this assay, both *Ppccd8* and *Ppmax2-1* mutants show a significant decrease of *caulonema* filaments number after (-)-GR24 application. In a separate experiment, a dose of 1 μ M of (-)-GR24 leads to opposite effects on *caulonema* filaments number of WT (increased) and *Ppccd8* (decreased) (Supplemental Figure IV-14 A, see also Figure IV-2), but has no significant effect on the *Ppmax2-1* mutant, though the same tendency to a decrease is observed. At this higher dose, the quintuple mutant affected in all five *PpKAI2L-A to E* genes shows a significant decrease of *caulonema* filaments number, like *Ppccd8*, and opposite to WT. Thus, similarly to *PpMAX2* loss of function, mutating clade (A-E) *PpKAI2L* genes does not abolish a response to the (-)-GR24 antiomer. A significant negative effect of (+)-GR24 on the number of filaments is observed for the quintuple *Ppkai2L a3*-b1-c3*-d3*-e2** mutant, as for WT and the *Ppccd8* and *Ppmax2-1* mutants (Figure IV-11 B). Thus, mutating any of clade (A-E) *PpKAI2L* gene does not hamper the response to (+)-GR24, and therefore likely the response to CCD8-derived compounds.

We then tested the effect of GR24 enantiomers on *Ppkai2L* mutants from the 3 other clades (Figure IV-11 C-E and Supplemental Figure IV-14). Strikingly, in control conditions, all mutants have more filaments than WT, as *Ppccd8*, except for clade (H,I,L) mutants which tend to have less filaments (Figure IV-11C-E and Supplemental Figure IV-14). Both the single mutant *Ppkai2L-j1** and the quintuple *Ppkai2L j1*-g1-m6*-i3*-l2* show a significant response to (-)-GR24 (less *caulonema*), as *Ppccd8* (Figure IV-11C and Supplemental Figure IV-14 A,B). Mutants with KO mutations in *PpKAI2L-F, -K, -H, -G, -M, -I* or *-L* show no clear response to (-)-GR24, as for WT.

When testing the response to (+)-GR24 (Figure IV-11 D-E and Supplemental Figure IV-14 C,D), the number of *caulonema* is clearly reduced in WT and *Ppccd8*, and in mutants carrying the *Ppkai2L \Delta h* mutation, alone or in combination with f^* , k^* i^* or l^* null mutations. Thus clade (F,K) and clade (H,I,L) genes do not play a role in the response to (+)-GR24. However, no more response to the (+)-GR24 enantiomer is observed for all mutants where the *PpKAI2L-J* gene is KO (Figure IV-11 D: $j1^*$ and $j1^*-g2^*-m2^*$, and Supplemental Figure IV-14 C,D). Interestingly, in the two mutants where *j* mutation is not null, but *PpKAI2L-G* gene is KO (7x and $j6-g5^*-m1$ mutants), the response to (+)-GR24 is also abolished (Supplemental Figure IV-14 C,D). Thus, from phenotypic assays on mutants' *caulonema*, both *PpKAI2L-J* and *-G* (and presumably *-M*) genes are likely involved in the response to (+)-GR24, and therefore the best candidates for receptors to PpCCD8-derived molecules.

To confirm that clade (J,G,M) PpKAI2L are likely receptors for PpCCD8-derived molecules, we tested the transcript levels of SL responsive genes in the corresponding mutants (Figure IV-12). We have shown previously that in WT and *Ppccd8* mutant, *PpKUF1LA* gene transcript abundance is increased 6 h after plant transfer on medium containing 3 μ M (±)-GR24, and that this response is enhanced in dark conditions (Lopez-Obando et al., 2018). We used this marker along with the *Pp3c6_15020* gene, previously found upregulated by (±)-GR24 (our unpublished data). Using GR24 enantiomers, we confirm the increase of both genes' transcript levels, following 1 μ M (+)-GR24 addition in WT and *Ppccd8*, but not in *Ppmax2-1*. Strikingly, an increase of transcript level following (-)-GR24 application is observed for both markers in the *Ppccd8* mutant, and for *PpKUF1LA* only in WT (Figure IV-12). In the quintuple mutant of clade (A-E), *PpKUF1LA* and *Pp3c6_15020* transcript levels are highly increased by the (+)-GR24 addition, but unchanged by (-)-GR24. In contrast, in the *Ppkai2L-j1** mutant, both genes transcript levels are increased by (-)-GR24 addition, and slightly increased (*PpKUF1LA*) or unchanged (*Pp3c6_15020*) by (+)-GR24 application. In the *Ppkai2Lj3-g3*-m1* mutant, the response markers transcript levels are slightly increased (*PpKUF1LA*) or unchanged by (+)-GR24 addition, while unchanged by (-)-GR24. Thus, the transcriptional response of the tested mutants confirms that clade (A-E) genes are not involved in the response to (+)-GR24, while this response is impaired in clade (J,G,M) mutants. Only *Ppccd8* and *Ppkai2L-j1** mutants show a clear and significant transcriptional response, with both markers, to (-)-GR24 addition.



Figure IV-12 – *Ppkai2L* mutant transcriptional response to (+)-and (-)-GR24. Transcript abundance analysis of the SL-responsive gene *PpKUF1LA* and *Pp3c6_15020*, in WT, *Ppccd8*, *Ppmax2-1* and *Ppkai2Like* mutants *a1-b1-c1-d1-e2** (clade (A-E)), j1* and j3-g3*-m1 (clade (J,G,M)), grown for 2 weeks in light, then transferred for 1 week in the dark, 6 hours after treatment with DMSO (control, grey plots), 1 μ M (+)-GR24 (cyan plots) or 1 μ M (-)-GR24 (red plots). Box plots of at least 4 biological repeats are shown, relative to mean (*PpAPT-PpACT3*) transcript abundance. 2-fold differences in median values of transcript levels between control and treated plants are estimated as significant (DE) and noted in corresponding colors (cyan for (+)-GR24 and red for (-)-GR24).

Discussion

Are PpCCD8-derived products non-canonical SLs?

The PpCCD8-derived products are germination stimulants of *P. ramosa* group 2a seeds, harvested in a hemp field, but do not induce the germination of *P. ramosa* group 1 seeds, collected from an oilseed rape field. Decker et al. (2017) also reported the induction of Orobanche ramosa (old denomination of Phelipanche ramosa) seed germination by P. patens exudates, however without specifying the origin of the tested population. Differences in root parasitic weeds susceptibility can be attributed to the chemical nature of host plant exudates (Yoneyama et al., 2018b). Our results suggest that the PpCCD8 products share similarities with hemp secondary metabolites. So far, no known canonical SL has been isolated from hemp (Huet et al., 2020). Since P. patens likely produces carlactone (Decker et al., 2017), but lacks a true MAX1 homolog (Proust et al., 2011), we can hypothesize that PpCCD8-derived compounds may correspond to non-canonical SLs, derived from carlactone or hydroxyl carlactones (Yoneyama, 2020). Indeed, among analogs showing bioactivity on *P. patens caulonema* length, we previously showed that GR5, a non-canonical analog, was as active as (±)-GR24 (Hoffmann et al., 2014). As mimics of SLs for the presented work, we used the (+)- and (-)-GR24 artificial analogs, available at the time of our study. It is to note that both isomers are active on P. ramosa group 1 and group 2a seeds. However, as GS, the (+)-GR24 isomer, which has a canonical SL structure, is similar to (\pm) -GR24, while the (-)-GR24 isomer is far less active (de Saint Germain et al., 2019). For future identification of PpCCD8-derived compounds, non-canonical SL analogs such as the recently described Methyl Phenlactonoates (Jamil et al., 2020) would certainly be more adapted.

Looking for the best mimic of SLs or KL

The (-)-GR24 analog has a non-natural configuration, meaning a configuration that has so far never been isolated from plant exudates, contrary to the (+)-GR24 enantiomer, that bears configurations as 5-deoxystrigol ((+)-5DS) and strigol-type canonical SLs (Scaffidi et al., 2014). In our bioassays on moss phenotype, CL application decreases the number of *caulonema* of both WT and *Ppccd8* mutant, in a dose-response manner. A similar (though much stronger) effect is observed with the (+)-GR24, that we thus consider as the best mimic of *P. patens* CCD8-derived compounds. It is not surprising that (+)-GR24 is more potent than CL, as assays are realized in a wet medium and natural SLs are described as far less stable than synthetic analogs in aqueous medium (Akiyama et al., 2010; Boyer et al., 2012). In contrast, the effects of (-)-GR24 are weak, not dose responsive, and sometimes opposite in WT versus *Ppccd8* mutant. Indeed, in several assays, we observed a significant increase of *caulonema* number in WT (Figure IV-2, Supplemental Figure IV-14A) while this number consistently decreases in *Ppccd8* (Figure IV-11, Supplemental Figure IV-14 A, B). Interestingly, we also observed an increase of *caulonema* number when testing KAR₂, though only significant at 10 µM on *Ppccd8*. So far, we had not observed any effect of karrikins (KAR₁) on *P. patens* phenotype (Hoffmann et al., 2014), and this is thus the first hint of a possible effect of some karrikins on moss, that needs to be confirmed. It is puzzling however that the effect of KAR₂ is better seen in *Ppccd8* (thus in the absence of SLs), while the effect of (-)-GR24 is better seen in WT versus Ppccd8. Thus, the (-)-GR24 is not very robust as a mimic of yet-to-be-identified moss KL. This could also suggest that the moss KL is somehow different from Angiosperm KL.

Biochemical characterization of the PpKAI2L proteins highlights those from the (A-E) subclade as KL receptors

In biochemistry experiments, among used analogs, we observed that the (-)-GR24 enantiomer is better bound by AtKAI2 and all tested PpKAI2L proteins, from (A-E) clade. This confirms a recent report (Bürger et al., 2019) of preferential binding of (-)-5DS by PpKAI2L-C, -D and -E. Surprisingly, the (+)-GR24 poorly interacts with these PpKAI2L proteins. As the (-)-GR24 enantiomer is a mimic of KL in Arabidopsis, this result suggests that clade (A-E) PpKAI2L proteins may share a perception mechanism with AtKAI2, and furthermore may recognize KL-like compounds. It is to note that AtKAI2 is not degraded following KAR perception (Waters et al., 2015a), and this could be tested on clade (A-E) PpKAI2L proteins. As to the two other tested clades (F,K) and (H,I,L), none of the interaction assays allowed to highlight a preferential binding of GR24 enantiomers. Unfortunately, none of the PpKAI2L proteins from clade (J,G,M) could be purified for interaction assays, as also experienced by Bürger *et al.* (2019). In the future, overexpression in *P. patens* or in other heterologous systems (yeast, insect cells) may be a solution to produce these proteins and permit their biochemistry studies.

PpKAI2L-H is the most efficient hydrolase among PpKAI2L proteins

The PpKAI2L-H protein shows a high cleavage activity towards all four GR24 stereoisomers as well as towards the synthetic probe (\pm)-GC242, compared to any other PpKAI2L protein, but also compared to Arabidopsis AtKAI2 and AtD14 proteins. Mutating the Leu28 residue into a Phe is enough to reduce the efficiency of the enzymatic activity (strong reduction of the k_{cat} and of the V_{max}), but has no effect on the $K_{1/2}$ towards (\pm)-GC242. The efficient cleavage activity of PpKAI2L-H is therefore likely not due to a stronger affinity of this protein for the substrate. This is in accordance with previous hypothesis that the Leu²⁸ residue (as the Phe¹⁸¹ residue), that is unique to PpKAI2L-H, does not particularly enlarge the pocket size of PpKAI2L-H (Bürger et al., 2019). Among other plant D14/KAI2 proteins, hydrolytic activity is observed for the pea PsD14/RMS3 towards three out of four GR24 enantiomers (this study, Figure 6A), and has also been reported for two KAI2 homologs, respectively from *Selaginella moellendorffii* (SmKAI2b, towards (+)-GR24) and *Marchantia polymorpha* (MpKAI2b, towards (-)-GR24 (Waters et al., 2015b)). The conserved strong enzymatic activity of PpKAI2L-H would thus have a specific (and ancient) role in plants, may be as a cleaning enzyme, to eliminate an excess of signaling molecules (Seto et al., 2019).

When mutating the *PpKAI2L-H* gene alone (Δh mutant), no striking phenotype is observed (Supplemental Figure IV-12B), and in particular, the phenotypic response to (+)-GR24, that mimics CCD8-derived compounds, is similar to that of WT plants (Figure IV-11D), contrary to other mutants. In red light however, the gametophores of Δh -*i2* and* Δh -*i3*-11* mutants are less elongated than WT gametophores, as it is also observed in *Ppccd8*. However, if, for *Ppccd8*, this can be related to the higher number of filaments, leading to the initiation of more (but smaller) gametophores, the number of filaments in the dark is not higher in clade (H,I,L) mutants. Even more, clade (H,I,L) mutants tend to have less filaments than WT (Figure IV-11D, E and Supplemental Figure IV-14A). Altogether, these phenotypes, although quite tenuous, could suggest that clade (H,I,L) genes may undertake a specific role in *P. patens* development. The relation of this role to PpKAI2L-H enzymatic activity remains to be discovered.

Arabidopsis Atd14-1 kai2-2 mutant complementation assay is more than anecdotal

Using the endogenous AtD14 promoter, we confirmed the incapacity of PpKAI2L-C and H proteins to fulfill Arabidopsis D14 function in rosette branching, previously reported using the 35S promoter (Bürger et al., 2019). We can extend this observation to PpKAI2L-D, PpKAI2L-F and PpKAI2L-G, never tested before for rosette branching complementation. Interestingly, one line expressing PpKAI2L-J shows partial complementation of the branching that could be the hint of SL perception function for this protein. However, given the phenotype of moss mutants in clade (J,G,M), one would have expected PpKAI2L-G expressing lines also to complement the branching phenotype, unless the PpKAI2L-J is more active than its counterparts from the same clade. Using the endogenous AtKAI2 promoter, we also confirmed as observed by Bürger et al. (2019), that PpKAI2L-C and -H proteins cannot complement the kai2-2 mutation effect, and extend this observation to PpKAI2L-D, and -J. However, expressing moss PpKAI2L-G reduces the size of Atd14-1 kai2-2 hypocotyls, suggesting that PpKAI2L-G may be able to perceive and transduce the endogenous KL signal, though it is not able to respond to (-)-GR24. Strikingly, when expressed under the control of AtKAI2 promoter, the AtD14 protein but also the moss PpKAI2L-C, -J or -H proteins in Arabidopsis Atd14-1 kai2-2 exacerbates the defect induced by the kai2-2 mutation by leading to even more elongated hypocotyls. This suggests a putative interaction of these proteins with the Arabidopsis KAI2/KL pathway, that needs further investigations. Even more strikingly, (+)-GR24-induced shorter hypocotyls are observed in PpKAI2L-H expressing lines, as in AtD14 expressing lines, which may indicate that the strong hydrolytic activity of PpKAI2L-H can fulfil AtD14 function in the seedling. Still, it is clear that none of the PpKAI2L protein fully complements the AtD14, nor the KAI2 function.

Genetic analysis suggests that genes from the (A-E) clade are likely involved in the PpMAX2 dependent pathway

Mutants phenotype clearly distinguish clade (A-E) from the three other clades. Indeed, the 5x PpKAI2L-A to -E mutant shows a phenotype in white light quite similar to that of *Ppmax2-1* mutant, as well as elongated gametophores under red light, and low number of *caulonema* filaments in the dark, suggesting that clade (A-E) PpKAI2L and PpMAX2 proteins could be part of a same pathway. As PpKAI2L proteins from the (A-E) clade preferentially bind the (-)-GR24 enantiomer, we expected the mutants in this clade to be blind to (-)-GR24 application. This is what we observe when testing the transcriptional response markers, which transcript levels are unchanged by (-)-GR24 application in both the Ppmax2-1 and the 5x (clade A-E) *Ppkai2l* mutant, while transcript levels of the response markers are increased in the *Ppccd8* mutant (but not in WT, Figure 12). However, as mentioned above, it should be noted that the (-)-GR24 is apparently not a good mimic of the unknown moss KL, and therefore other transcriptional response markers need to be found, that would reflect the moss KL response, as DLK2 or STH7 for Arabidopsis KL (Nelson et al., 2010; Waters and Smith, 2013). As for the phenotypic response, application of 0.1 µM (-)-GR24 had no effect on clade (A-E) mutants caulonema number in the dark, nor on WT, but significantly decreased both Ppccd8 and Ppmax2-1 caulonema number (Figure IV-11A). Strikingly, a 1 µM concentration of (-)-GR24, that has opposite effects on WT (increases the number of *caulonema*) and *Ppccd8* (decreases the number of *caulonema*), led to no response of the *Ppmax2-1* mutant, while the 5x (clade A-E) mutant showed a significant decrease of *caulonema* number (Supplemental Figure IV-14). Thus, the 5x (clade A-E) mutant is still able to perceive (-)-GR24, as well as the Ppmax2-1 mutant. This does not rule out the hypothesis of clade (A-E) PpKAI2L and PpMAX2 proteins being in a same pathway, but further indicates that the (-)-GR24 enantiomer is a poor mimic of a moss KL. In addition, a dual effect of (-)-GR24, promoting both the KAI2 and the D14 pathways has been reported in Arabidopsis roots (Villaecija-Aguilar et al., 2019), and could explain the residual response of the clade (A-E) *Ppkai2l* mutant, through other PpKAI2L proteins, in a PpMAX2 independent pathway.

Finally, both phenotypic response in the dark and transcriptional response to the (+)-GR24 enantiomer are unaffected in clade (A-E) *Ppkai2l* mutant, indicating that clade (A-E) *PpKAI2L* proteins are likely not receptors to CCD8-derived compounds.

PpKAI2L- J, -G, -M mediate PpCCD8 derived (SL-related) response

In white light conditions (Figure IV-9), mutants affecting other clades than (A-E) show either similar to WT phenotype (mutants affecting clade (H,I,L) genes), or intermediate phenotype between WT and *Ppccd8* (mutants in clade (F,K) and (J,G,M) clades). In the dark (Figure IV-11), the caulonema number in control conditions is also intermediate between WT and *Ppccd8* mutant for mutants in clades (F,K) and (J,G,M), while it is similar to WT (or slightly smaller) in clade (H,I,L) mutants. Based on the hypothesis that synthesis and response mutants show similar phenotypes, genes from clades (F,K) and (J,G,M) are thus the best candidates for PpCCD8-derived compounds receptors. When testing the phenotypic response of these mutants to (+)-GR24 application, plants with KO mutations for *PpKAI2L-J* or *PpKAI2L-G/M* (Figure IV-11, $j1^*$, and $j1^*-g2^*-m2^*$, and Supplemental Figure IV-14, $j7^*-g1-m1$, i8*-g1 - m5*, and i6-g5*-m1) no more respond to this compound. In contrast, both $\Delta h-f1*-k1*-i4$ and $\Delta h-f3*-k3*-i6$ mutants, show a significant response to (+)-GR24 application (Figure IV-11 and Supplemental Figure IV-14), indicating that KO mutations in both *PpKAI2L-F* and *PpKAI2L-K*, or deletion of *PpKAI2L-H* do not abolish the response to the CCD8-derived compound mimic, not even additively. The absence of response in higher order mutants where either *PpKAI2L-J* or *PpKAI2L-G* are KO confirms the prominent role of both genes in the response to (+)-GR24. However, if, as expected, transcript levels of the $Pp3c6_{15020}$ response marker gene are unchanged in both $j1^*$ and $j3-g3^*-m1$ mutants following (+)-GR24 application (Figure IV-12), transcript levels of the *PpKUF1LA* gene are increased in both mutants, suggesting a response to the SL analog. Thus, while the KO mutation of either *PpKAI2L-J* or *PpKAI2L-G* is sufficient to abolish the phenotypic response in the dark, mutations in both genes are necessary to completely abolish the transcriptional response to (+)-GR24. The transcriptional markers first identified using (\pm) -GR24 (Lopez-Obando et al., 2016a, 2018) may not be fully specific to assay the response to enantiomers. It could also suggest that the transcriptional response, which is assessed far earlier than the phenotypic response (6 hours versus 15 days), is perhaps more sensitive to a very slight activation of the CCD8-compound pathway by PpKAI2L proteins.

PpKAI2L proteins are likely receptors in two separate pathways, respectively dependent and independent from the PpMAX2 F-box protein.

Our previous results on the PpMAX2 F-box protein indicated that, in contrast to its homolog in flowering plants, it is not involved in response to CCD8-derived compounds (Lopez-Obando et al., 2018). Like MAX2 in flowering plants however, PpMAX2 plays a role in gametophore early development and photomorphogenesis. We suggested that PpMAX2 could play a role in the moss KL signalling pathway, but we lacked evidence for other actors in this pathway in *P. patens*. The present work indicates that PpKAI2L-A to -E are α/β hydrolases involved in the same pathway as

PpMAX2, since mutating these genes lead to similar light-related phenotypes as those of *Ppmax2* mutant. Specific mimics for the moss KL are however still missing for further evidence that PpKAI2L-A-E are receptors of the moss KL. Still, these results are consistent with the view that the KL pathway is ancestral relative to the SL pathway, and that the ancestral role of MAX2 in the land plants lineage is the transduction of the KL signal (Bythell-Douglas et al., 2017; Walker et al., 2019).

As to SL-related pathway, PpKAI2L-J and PpKAI2L-G proteins are likely receptors to CCD8-compounds, which we suspect to be non-canonical SLs. Strikingly, these receptors are not particularly closer to D14 than other PpKAI2L proteins. As hypothesized earlier by Lopez-Obando (2016) and Bythell-Douglas (2017), the expansion of the PpKAI2L family might have enabled in moss (and not in other bryophytes as *Marchantia polymorpha*, that counts 2 *MpKAI2* genes), as in parasitic Angiosperms (Conn et al., 2015; Toh et al., 2015; de Saint Germain et al., 2020)), the emergence of SL sensitivity. Neofunctionalization of additional KAI2 copies in *P. patens* ancestry towards SL perception is therefore a possible explanation for what we observe in this moss and would reveal a convergent evolution process, relative to the emergence of D14 in seed plants. We can also imagine that these neo-functionalized PpKAI2L lost the ability to interact with MAX2 in *P. patens* and established a different protein network that potentially integrates new factors like an alternative F-box, since PpMAX2 is not necessary for SL sensitivity. The remaining question is therefore to determine how the SL signal is transduced downstream of perception by PpKAI2L-J -G -M.

Consequently, the search for interactants to these moss KL and CCD8-derived compounds receptors should be a priority in the next future. As SMXL proteins are key players of both SL and KL pathways in flowering plants (Soundappan et al., 2015; Wang et al., 2015b; Khosla et al., 2020) specific involvement of PpSMXL homologs (4 genes) was also examined (chapters VI and VII).

Five more PpKAI2L proteins are present and expressed in moss (Lopez-Obando et al, 2016), and mutant analyses indicate that those are neither KL nor CCD8-derived compounds receptors. Three of them however, among which the efficient hydrolase PpKAI2L-H, are likely involved in moss development, through pathways that remain to be discovered.

Methods

Plant Materials and Growth Conditions. The *Physcomitrium (Physcomitrella) patens* Gransden wild-type (WT) strain was used and grown as previously described (Hoffmann et al, 2014; Lopez-Obando et al, 2018), at 24°C in long day (16h) conditions, (22°C 8h night), except for assays in red light. Unless otherwise explicitly stated in legends, experiments were always carried out on PpNO₃ medium (corresponds to the minimal medium described by Ashton et al., 1979), in the following control conditions: 25°C during daytime and 23°C at night, 50% humidity, long days conditions with 16 hours of day and 8 hours of night (quantum irradiance of ~80 μ mol/m²/s). Multiplication of tissues from young *protonema* fragments prior to every experiment is done in the same conditions but using medium with higher nitrogen content (PpNH₄ medium, PpNO₃ medium supplemented with 2.7 mM NH₄ tartrate). For red light experiments, plants were grown on PpNO₃ medium in Magenta pots at 25°C, in continuous red-light (~45 μ mol μ mol/m²/s).

Germination assay on root parasitic plant seeds. *P. patens* WT plants were grown on PpNH₄ plates with cellophane disks for 2 weeks then the plants (and cellophane) were transferred on PpNO₃ medium without phosphate (Phosphate buffer was replaced by 1g/L of MES buffer and the pH adjusted to 5.8) for another 2 week. Moss exudates were collected by transferring the plants (still on cellophane disks) on plates with 10 mL distilled water, placed in growth chamber with gentle agitation. After 48h, exudates were pipeted and filtered ($0.2 \mu m$). Exudates were diluted twice prior testing their germination stimulant activity on preconditioned seeds of parasitic plants, as described previously (Pouvreau et al., 2013). Distilled water was used as control. For germination assays on plates (Figure 1B-C), WT and *Ppccd8* mutant were cultivated as above, and *P. ramosa* seeds were added on the plates after 10 days of phosphate starvation. Seeds were counted out of 3 plates, with 7-10 microscope fields per plate.

CRISPR-Cas9 mediated mutagenesis and homologous recombination. P. patens mutants were obtained as described in Lopez-Obando et al (2016a), using CRISPR-Cas9 technology. Coding sequences of *PpKAI2L* were used to search for CRISPR RNA (crRNA) contiguous to a PAM motif recognized by Streptococcus pyogenes Cas9 (NGG), using the webtool CRISPOR V4 against P. patens genome Phytozome V9 (http://crispor.tefor.net/). crRNAs located in the first third of the coding sequence, with highest possible specificity score, and fewest possible predicted off-targets, were selected. Small constructs containing each crRNA fused to either the proU6 or the proU3 snRNA promoter in 5' U3 or U6 promoter (Collonnier et al., 2017), and to the tracrRNA in 3', encased between attB1/attB2 GateWay recombination sequences, were synthesized by Twist Biosciences. These inserts were then cloned into pDONR207 vectors. Polyethylene glycol-mediated protoplast transformation was performed with multiple pDONR207-sgRNA according to the protocol described by (Lopez-Obando et al., 2016b). Mutations of the *PpKAI2L* genes were confirmed by PCR amplification of *PpKA12L* loci around the recognition sequence of each guide RNA and sequencing of the PCR products. The deletion mutant in PpKAI2L-H (Δh) was obtained through homologous recombination. The full coding sequence of PpKAI2L-H from ATG to stop was replaced by a resistance cassette. A 550 bp PpKAI2L-H 5' CDS flanking sequence was cloned into the pBNRF vector (Thelander et al., 2007) cut with BstBI/XhoI. Then a 500 bp PpKAI2L-H 3' CDS flanking sequence was cloned into the BNRF-PpKAI2L-H 5' construct digested with BcuI, so that the kanamycin resistance cassette of the vector was surrounded by PpKAI2L-H 5' and 3' flanking sequences. Moss WT protoplasts were transformed with the resulting construct as described previously (Lopez-Obando et al., 2016b), and transformants selected on 50mg/L Geneticin/G418. Transient expression of the CRE recombinase (Trouiller et al., 2006) in a confirmed transformant allowed to remove of the resistance cassette and to obtain the *Ppkai2l-\Delta h* mutant, as described Figure IV-8 and Supplemental Figure IV-12.

Phenotypic assays on moss. Analysis of *caulonema* growth in the dark was performed in 24-well plates, starting from very small pieces of protonema, with \sim 2 weeks of growth in control conditions before incubation (± treatment) in the dark and placed vertically for \sim 10 days (See also Guillory and Bonhomme, contribution to the book "Methods in strigolactone research" in annex).

Chemicals. Racemic and pure enantiomers of GR24, (\pm) -GC242 probe were produced by F-D Boyer (ICSN, France). Racemic Z-CL was kindly provided by A. Scaffidi (University of Western Australia, Perth, Australia). Chemicals were diluted in DMSO or acetone as indicated in legends to figures. KAR₂ was purchased from Chiralix.

RT-qPCR analysis. Freshly grinded WT (Gransden) tissues were inoculated in Petri dishes of PpNO₃ medium, overlaid with a cellophane sheet. Protonema tissues were harvested after 6 days, 10 days or 15 days of growth in long days conditions (25°C during the day and 22°C during the night, 50% hygrometry, quantum irradiance of 75 µmol m⁻² s⁻¹). To obtain older gametophores and spores, WT were regenerated from spores for approximately two weeks and then transferred to Magenta pots containing PpNO₃ medium (9 plants per pot) and cultivated in the same conditions as written above. Gametophores were harvested after 35 days and then from different plants after 70 days. After 70 days, rhizoids were also harvested after being separated from gametophores' shoots by dissection. Both protonema and gametophores samples were immediately flash frozen in liquid nitrogen and kept at -80°C until RNA extraction. Remaining pots were transferred at 35 days to short days conditions (15°C, 100% hygrometry, quantum irradiance of 15 µmol m⁻² s⁻¹) for approximately two months until capsule maturity. Capsules were sterilized (90% chlore and 10% pure ethanol) then rinsed with sterile water. Each of the four biological replicates consisted of 10-20 capsules from which spores were freed by mechanical disruption and separated from capsules' debris by filtering through a 25µm nylon mesh. Spores were kept in sterile water and flash frozen in liquid nitrogen and kept at -80°C until RNA extraction. For all samples except for spores, tissues were grinded in liquid nitrogen using a mortar and pestle and RNA were extracted and subsequently treated with DNAseI using the Plant RNeasy Mini extraction kit (Qiagen) following the manufacturer's instructions. Spores were recovered in 1mL TRIzol reagent (Invitrogen) and crushed manually using fine pestles. RNA was separated from cell debris and protein using chloroform and then precipitated with isopropanol and washed with ethanol 70%. RNA pellets were dissolved in RLT buffer from the Qiagen Plant RNeasy Mini kit and treated with DNAse I on columns following the manufacturer's instructions. 500 ng of each RNA sample was used for retro-transcription using the RevertAid H Minus Reverse Transcriptase from Thermo Fisher. Quality of obtained cDNA extracts was checked by semi-quantitative RT-PCR using the reference gene *PpAPT*. Quantitative RT-PCR were carried out in 5µL using the SsoAdvanced Universal SYBR Green Supermix from BioRad and the following program on QuantStudio™ 5 (ThermoFisher): initial denaturation at 95°C for 3 minutes, then 45 cycles with 95°C 10 seconds and 60°C 30 seconds. Using the CTi (for the genes of interest) and CTref (mean for the 2 reference genes) values obtained, relative expression is given by $RE = 2^{-CTi}/2^{-CTref}$. Note that in Supplemental Figure IV-3 the values given are Log (RE).

Constructs, generation of transgenic lines. The expression vectors for transgenic *Arabidopsis* were constructed by MultiSite Gateway Three-Fragment Vector Construction kit (Invitrogen). All the PpKAI2L constructs were tagged with 6xHA epitope tag at their C-terminus. Lines were resistant to hygromycin. AtD14 native promoter (0.8 kb) and AtKAI2

native promoter (0.7 kb) were amplified by PCR from Col-0 genomic DNA and cloned into *pDONR-P4P1R*, using Gateway recombination (Invitrogen) (see Supplementary Table IV-2 for primers). AtD14 CDS and AtKAI2 CDS were PCR amplified from Col-0 cDNA, PpKAI2cds were PCR amplified from *Physcomitrella patens* cDNA and recombined into *pDONR221* (Invitrogen). *6xHA* with a linker (gift from U. Pedmale) was cloned into *pDONR-P2RP3* (Invitrogen). The suitable combination of promoters, CDS and 6xHA was cloned into the *pH7m34GW* final destination vectors by using three fragments recombination system (Karimi et al., 2007), and named pD14::cds-6xHA or pKAI2::cds-6xHA. Transformation of *Arabidopsis Atd14-1kai2-2* double mutant, Landsberg background (gift from M. Waters) was performed according to the conventional dipping method (Clough and Bent, 1998), with Agrobacterium strain GV3101. For all constructs, more than 12 independent T1 lines were isolated and between 2 to 4 representative single-insertion lines were selected in T2. Only 2-3 lines per constructs were shown in these analyses. Phenotypic analysis shown in Figure IV-7 and Supplementary Figure IV-10 were performed on T3 homozygous segregating lines.

Arabidopsis hypocotyl elongation assays. Arabidopsis seeds were surface sterilized by consecutive treatments of 5 min 70% (v/v) ethanol with 0.05% (w/v) sodium dodecyl sulfate (SDS) and 5 min 95% (v/v) ethanol. Then seeds were sown on 0,25 X Murashige and Skoog (MS) media (Duchefa Biochemie) containing 1% agar, supplemented with 1 μ M (+)-GR24, (-)-GR24 or with 0.01 % DMSO (control). Seeds were stratified at 4 °C (2 days in dark) then exposed to white light for 3 h, transferred to darkness for 21 h, and exposed to low light for 4 days at 21°C. Plates were photographed and hypocotyl lengths were quantified using ImageJ (http://imagej.nih.gov/ij/).

Arabidopsis branching quantification. Experiments were carried out in summer in greenhouse., under long photoperiods (15–16 h per day); daily temperatures fluctuated between 18°C and 25°C. Peak levels of PAR were between 700 and 1000 μ mol m⁻² s⁻¹. Plants were watered twice a week with tap water. The number of rosette leaves was counted just after bolting of the main shoot, and the number of rosette branches longer than 5 mm was counted when the plants were 40 days old.

Expression and purification of AtD14, RMS3 and AtKAI2, with cleavable GST tag was performed as described in de Saint Germain et al, 2016. For PpKAI2L proteins expression, the full-length coding sequences from Physcomitrella patens were amplified by PCR using cDNA template and specific primers (see Supplementary Table IV-1) containing a protease cleavage site for tag removal, and subsequently cloned into the pGEXT-4T-3 expression vector. For the PpKAI2L-L, the N-terminal 47 amino acids have been removed. The expression and purification of PpKAI2 proteins followed the same method as for AtD14 and AtKAI2.

Site-directed mutagenesis. Site-directed mutagenesis experiments were performed using QuickChange II XL Site Directed Mutagenesis kit (Stratagene), performed on pGEX-4T-3-PpKAI2L-H (see Supplementary Table IV-1 for primers). Mutagenesis was verified by systematic DNA sequencing.

Enzymatic degradation of GR24 isomers by purified proteins. The ligand (10 μ M) was incubated without and with purified RMS3/AtKAI2/PpKAI2L proteins (5 μ M) for 150 min at 25 °C in PBS (0.1 mL, pH = 6.8) in presence of (±)-1-indanol (100 μ M) as internal standard. The solutions were acidified to pH = 1 by addition of trifluoroacetic acid (2 μ L) to quench the reaction and centrifugated (12 min, 12,000 tr/min). Thereafter the samples were subjected to RP-UPLC-MS analyses. The instrument used for all the analysis was an Ultra Performance Liquid Chromatography system

equipped with a PDA and a Triple Quadrupole mass spectrometer Detector (Acquity UPLC-TQD, Waters, USA). RP-UPLC (HSS C₁₈ column, 1.8 μ m, 2.1 mm × 50 mm) with 0.1% formic acid in CH₃CN and 0.1% formic acid in water (aq. FA, 0.1%, v/v, pH 2.8) as eluents [10% CH₃CN, followed by linear gradient from 10 to 100% of CH₃CN (4 min)] at a flow rate of 0.6 mL/min. The detection was performed by PDA and using the TQD mass spectrometer operated in Electrospray ionization positive mode at 3.2 kV capillary voltage. The cone voltage and collision energy were optimized to maximize the signal and was respectively 20 V for cone voltage and 12 eV for collision energy and the collision gas was argon at a pressure maintained near of 4.5.10⁻³ mBar.

Enzymatic assay with pro-fluorescent probes and *p***-nitrophenyl acetate** has been performed as described in (de Saint Germain et al., 2016), using a TriStar LB 941 Multimode Microplate Reader from *Berthold Technologies*.

Temperature melts proteins.

Differential Scanning Fluorimetry (DSF) experiments were performed on a CFX96 TouchTM Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, California, USA) as described in de Saint Germain et al 2016. *nanoDSF*. Proteins were diluted in Phosphate buffer saline (PBS) (100 mM Phosphate, pH 6.8, 150 mM NaCl) to ~10 μ M concentration. Ligand was tested at the concentration of 200 μ M. The intrinsic fluorescence signal was measured as a function of increasing temperature in Prometheus NT.48 fluorimeter (NanotemperTM), with 55% excitation light intensity and 1 °C/minute temperature ramp. Analyses were performed on capillaries filled with 10 μ L of respective samples. Intrinsic fluorescence signal expressed by the 350 nm/330 nm emission ratio, which increases as the proteins unfold, is plotted as a function of temperature. The plots are one of the three independent data collections that were performed for each protein.

Intrinsic tryptophan fluorescence assays and determination of the dissociation constant K_D has been performed as described in (de Saint Germain et al., 2016), using Spark[®] Multimode Microplate Reader from Tecan.

Direct ESI-MS in denaturant conditions. Mass spectrometry measurements were performed with an electrospray Q-TOF mass spectrometer (Waters) equipped with the Nanomate device (Advion, Inc.). The HD_A_384 chip (5 μ m I.D. nozzle chip, flow rate range 100–500 nL/min) was calibrated before use. For ESI–MS measurements, the Q-TOF instrument was operated in RF quadrupole mode with the TOF data being collected between m/z 400–2990. Collision energy was set to 10 eV and argon was used as collision gas. Mass spectra acquisition was performed after denaturation of PrKAI2 \pm ligand in 50% acetonitrile and 1% formic acid. The Mass Lynx 4.1 (Waters) and Peakview 2.2 (Sciex) softwares were used for acquisition and data processing, respectively. Deconvolution of multiply charged ions was performed by applying the MaxEnt algorithm (Sciex). The protein average masses are annotated in the spectra and the estimated mass accuracy is \pm 2 Da. External calibration was performed with NaI clusters (2 μ g/ μ L, isopropanol/H₂O 50/50, Waters) in the acquisition m/z mass range.

Localization of the fixation site of ligands on PpKAI2L. PpKAI2L-ligand mixtures were incubated for 10 min before to be submitted overnight to Glu-C proteolysis. Glu-C-generated peptides mixtures were analyzed by nanoLC-MS/MS with the Triple-TOF 4600 mass spectrometer (AB Sciex) coupled to the nanoRSLC ultra performance liquid chromatography (UPLC) system (Thermo Scientific) equipped with a trap column (Acclaim PepMap 100 C₁₈, 75 μ m i.d. × 2 cm, 3 μ m) and an analytical column (Acclaim PepMap RSLC C₁₈, 75 μ m i.d.× 25 cm, 2 μ m, 100 Å). Peptides

were loaded at 5 μ L/min with 0.05% TFA in 5% acetonitrile and peptides separation was performed at a flow rate of 300 nl.min⁻¹ with a 5 to 35% solvent B gradient in 40 min. Solvent A was 0.1% formic acid in water, and solvent B was 0.1% formic acid in 100% acetonitrile. NanoLC-MS/MS experiments were conducted in a Data Dependent acquisition method by selecting the 20 most intense precursors for CID fragmentation with Q1 quadrupole set at low resolution for better sensitivity. Raw data were processed with MS Data Converter tool (AB Sciex) for generating .mgf data files and protein identification was performed using the MASCOT search engine (Matrix Science, London, UK) against the PrKAI2 sequence with oxidation of methionines and ligand-histidine adduct as variable modifications. Peptide and fragment tolerance were respectively set at 20 ppm and 0.05 Da. Only peptides with a mascot ion score above identity threshold (25) calculated at 1% FDR

Homology model. Superimposition model figure were prepared by using PyMOL (DeLano Scientific) with the crystal structure of AtD14 (PDB ID 4IH4) and PpKAI2L-H (PDB ID 6AZD)

Phylogenetic analysis. Phylogenic analysis was conducted in MEGA5 (Tamura et al., 2011). Protein sequences were aligned using CLUSTALX. The Maximum Likelihood method based on the Dayhoff matrix-based model was used (Schwarz and Dayhoff, 1979). Initial tree(s) for the heuristic search were obtained automatically as follows. When the number of common sites was < 100 or less than one fourth of the total number of sites, the maximum parsimony method was used; otherwise BIONJ method with MCL distance matrix was used.

Statistical analyses. Kruskal-Wallis, Mann-Whitney and *post-hoc* Dunn multiple comparisons tests (details in figures legends) were carried out either in R 3.6.3 or in GraphPad Prism 8.4.2, except for Figures IV-11 and Supplemental Figure IV-14, see the legends. Unless otherwise defined, used statistical significance scores are as follow: $\# 0.05 \le p < 0.1$, $* 0.01 \le p < 0.05$, $** 0.001 \le p < 0.01$, *** p < 0.001. Same letters scores indicate that $p \ge 0.05$ (non-significant differences).

Supplemental data

Supplemental Figure IV-1. Phenotypic response to GR24 enantiomers and KAR₂: length of *caulonema* filaments.

Supplemental Figure IV-2. Sequence alignment of *Physcomitrium patens* (Pp) protein with D14 and KAI2 proteins from *Arabidopsis thaliana* (At).

Supplemental Figure IV-3. Expression of *PpKAI2L* genes along *P. patens* vegetative development.

Supplemental Figure IV-4. eFP-Browser expression data of *PpKAI2L* genes.

Supplemental Figure IV-5. Biochemical analysis of the interaction between PpKAI2 proteins and the GR24 isomers ligands by DSF.

Supplemental Figure IV-6. Intrinsic tryptophan fluorescence of PpKAI2L-D (A-D), PpKAI2L-H (E-H), PpKAI2L-H (I-L), PpKAI2L-F (M-P), PpKAI2L-K (M-P), AtKAI2L-K (U-X) and AtD14 (Y-AB) proteins in the presence of SL analogs.

Supplemental Figure IV-7. PpKAI2L hydrolysis activity towards p-NPA.

Supplemental Figure IV-8. Characterization of (±)-GC242 profluorescent probe activity on moss.

Supplemental Figure IV-9. Mass spectrometry characterization of covalent PpKAI2-ligand complexes.

Supplemental Figure IV-10. Complementation assays of Arabidopsis Atd14-1 kai2-2 double mutant.

Supplemental Figure IV-11. Extra mutations obtained in *PpKAI2-Like* genes.

Supplemental Figure IV-12. Ppkai2L- Δh mutant and phenotype of *Ppkai2L* mutants in light.

Supplemental Figure IV-13. Gametophores of *Ppkai2L* mutants in red light.

Supplemental Figure IV-14. Phenotypic response of *Ppkai2L* mutants to (-)-GR24 and (+)-GR24 application: *caulonema* number in the dark.

Supplementary table IV-1. Oligonucleotides used in this study.

Supplementary table IV-2. Mutants used in the study.

Supplementary table IV-3. List of gene sequences used in this study.



Supplemental Figure IV-1 - Phenotypic response to GR24 enantiomers and KAR₂: length of *caulonema* filaments. Maximal length of *caulonema* filaments was measured in WT (A) and *Ppccd8* SL synthesis mutant (B) grown 10 days vertically in the dark, following application of increasing concentrations of (+)-GR24, (-)-GR24 and KAR₂, as indicated. Control = DMSO. Significant differences between control and treated plants within a genotype based on a Kruskal-Wallis test (Dunn *post-hoc*): ***, p < 0.001; **, p < 0.01; *, p < 0.05; # p < 0.1. For each genotype and treatment, n = 24 plants grown in 3 different 24 well-plates.



Supplemental Figure IV-2 - Sequence alignment of *Physcomitrium patens* (Pp) protein with D14 and KAI2 proteins from *Arabidopsis thaliana* (At). Three amino acid residues corresponding to the catalytic triad are marked with stars. Amino acid residues interacting with the GR24 analogs in the binding pocket are indicated with a blue arrowhead. Amino acid numbers are indicated for AtD14. The secondary structure assignment is based on the crystal structure of AtD14 and the labels of the major strands and helices are based on Yao et al. 2016.



Supplemental Figure IV-3 - Expression of *PpKA12L* **genes along** *P. patens* **vegetative development.** Relative expression is given as $ln(2^{-Cti}/2^{-mCtref})$, where mCtref is the mean of Ct values for two reference genes (*PpAPT*, *Pp3c8_16590* and *PpACT3*, *Pp3c10_17080*), for each biological replicate. 4 biological replicates and 2 technical repeats are included in the analysis for each gene and tissue. For each technical repeat, normalization was carried out using the mean of the expression of the two reference genes. (A) Comparison of mean values (error bars represent standard errors)

amongst all *PpKAI2L* genes. (B-E) Comparison amongst each of the 4 different subclades. When relevant, results of statistical analyses between tissues for a given gene (Kruskal-Wallis, p < 0.05) are indicated with bold letters. Samples recorded as *PpKAI2L-G* actually represent a mix of *PpKAI2L-G* and *PpKAI2L-M* transcripts, as their transcripts are almost identical.



Supplemental Figure IV-4 - eFP-Browser expression data of *PpKAI2L* **genes.** Expression levels are shown in a color scale, relative to the expression of the *PpAPT* reference gene. Diagrams were taken from *www.bar.utoronto.ca* in May 2018. Owing to the extreme similarity of *PpKAI2L-G* and *PpKAI2L-M* transcripts, they could not be singled out in the data set of Ortiz Ramirez *et al.* (2016).



Supplemental Figure IV-5 - Biochemical analysis of the interaction between PpKAI2 proteins and the GR24 isomers ligands by DSF. The melting temperature curves of AtKAI2 (A), AtD14 (B), PpKAI2L-C (C), PpKAI2L-D (D), PpKAI2L-E (E), PpKAI2L-F (F), and PpKAI2L-H (G) at 10 μ M, 200 μ M with (+)-GR24 (blue) and (-)-GR24 (red), and without ligand (black) are shown as assessed by DSF. Each line represents the average protein melt curve for three technical replicates and the experiment was carried out twice.





Supplemental Figure IV-6 - Intrinsic tryptophan fluorescence of PpKAI2L-D (A-D), PpKAI2L-H (E-H), PpKAI2L-H (I-L), PpKAI2L-F (M-P), PpKAI2L-K (M-P), AtKAI2L-K (U-X) and AtD14 (Y-AB) proteins in the presence of SL analogs. Changes in intrinsic fluorescence emission spectra of PpKAI2L proteins, in the presence of (+)-GR24 (A;E;I;M;Q;U;Y),(-)-GR24 (B;F;J;N;R;V;Z),various concentrations of (+)-2'-*epi*-GR24 (C;G;K;O;S;W;AA), (-)-2'-epi-GR24 (D;H;L;P;T;X;AB). Proteins (10 µM) were incubated with increasing amounts of ligand (0–800 µM, top line to bottom line, respectively). The observed relative changes in intrinsic fluorescence were plotted as a function of SL analog concentration and transformed to degree of saturation and used to determine the apparent K_D values relevant to Figure IV-5. The plots represent the mean of two replicates and the experiments were repeated at least three times. The analysis was performed with GraphPad Prism 8.0 Software.



Supplemental Figure IV-7 - PpKAI2L hydrolysis activity towards *p*-NPA. (A) Progress curves during the 4nitrophenyl acetate (*p*-NPA) (1 mM) hydrolysis by AtKAI2, AtD14 and PpKAI2L proteins (4 μ M). The release of 4nitrophenol was monitored (A₄₀₅) at 25 °C. (B) Michaelis-Menten plot of AtKAI2, AtD14 and PpKAI2L proteins steady state kinetics reaction velocity with *p*-NPA. Initial velocity was determined with *p*-NPA concentration from 15 μ M to 2000 μ M and protein at 4 μ M. Error bars represent SE of the mean of three replicates and the experiments were repeated at least two times. (C) Table: Kinetic constants of *p*-NPA toward PpKAI2 proteins. *K*_M and *k*_{cat} are steady-state kinetic constants and values represent the mean ± SE of three replicates.


Supplemental Figure IV-8 - Characterization of (±)-GC242 profluorescent probe activity on moss. (A) (±)-GC242 effect on moss caulonema number. Caulonema number measurements in the dark in the WT and *Ppccd8* SL synthesis mutant, following application of 1 μ M (±)-GR24 (yellow) and increasing concentrations of (±)-GC242. Ctl, control (same amount of DMSO). Mean of 3 biological repeats; n = 9-10 in each repeat. Statistical groups (comparison of all genotypes and treatments) are indicated by letters and were determined with a Kruskal-Wallis test followed by a Dunn *post hoc* test (p < 0.05). (B) Kinetic constants of AtD14, PpKAI2L-H, PpKAI2L-H^{L28F} towards (±)-GC242. $K_{1/2}$ and k_{cat} are pre-steady-state kinetic constants. $K_{1/2}$ and k_{cat} values represent the mean ± SE of three replicates.



Supplemental Figure IV-9 - Mass spectrometry characterization of covalent PpKAI2-ligand complexes.

Deconvoluted electrospray mass spectra of PpKAI2 protein before (left column) and after adding (-)-GR24 (middle column) or (+)-GR24 (right column). Peaks with an asterisk correspond to PpKAI2 covalently bound to a ligand. Mass increments are measured for different PrKAI2-ligand complexes: 96.3 Da for (+)-GR24 and (-)-GR24.



Supplemental Figure IV-10 - Complementation assays of Arabidopsis At*d14-1 kai2-2* **double mutant.** (A) Results of PCR amplification of CDS from transcripts of Arabidopsis transformed plants (homozygous T3 generation), as

indicated. All samples were checked for presence of transcripts. (B) Hypocotyl length of Ler (WT), *kai2-2*, At*d14-1 kai2-2* mutant (in Ler), and At*d14-1 kai2-2* mutant transformed using AtKAI2 promoter to control *AtD14*, *AtKAI2* (controls, same as shown figure 7B) or *PpKAI2L-C* and *-D* genes as noted below the graph. Hypocotyl length under low light, on ½ MS medium with DMSO (control, grey bars) 1 μ M (+)-GR24 (blue bars) or 1 μ M (-)-GR24 (red bars). Different letters indicate significantly different results between genotypes in control conditions based on a Kruskal–Wallis test (p < 0.05, Dunn *post hoc* test with p values corrected following the Benjamini-Hochberg method). Symbols in blue and red give the statistical significance of response to (+)-GR24 and (-)-GR24 respectively (Mann-Whitney tests, * 0.01 ≤p < 0.05, *** p ≤ 0.001).



Supplemental Figure IV-11 - Extra mutations obtained in *PpKAI2-Like* genes. WT nucleotidic and proteic sequences are shown, above altered sequences found in various CRISPR-Cas9 lines (in italics, numbered). The number of first shown amino-acid (aa) and the predicted secondary structure are indicated above the WT proteic sequence. The sgRNA sequence is shown in blue, with the PAM site underlined. Deletions are shown as dashes, insertions are noted with orange letters. The mutation type is shown on the right. Premature STOP codons are noted in bold, and with a red star on the aa sequence. On proteic sequences, the number of not shown aa is noted between slashes. For *PpKAI2L-E*, the serine (S) of the catalytic triad is noted in bold blue. For *PpKAI2L-H*, a deletion of the full coding sequence between ATG and STOP was obtained through homologous recombination; the use of CRE recombination led to 46 residual nucleotides, (not shown) corresponding to the LoxP site (see Methods). See Supplemental Table IV-2 for the list of mutants carrying one or several of the shown mutations.



Supplemental Figure IV-12 - Ppkai2L- Δh mutant and phenotype of *Ppkai2L* mutants in light. (A) Checking of the deletion mutant in PpKAI2L-H obtained through Homologous Recombination. Primers used for PCR shown are indicated with blue arrows. PCR was led on WT and Δh genomic DNA. Sequence of the deletion site is shown on Figure IV-8. (B) *Ppkai2L* mutant's 10-day-old phenotype in light conditions-scale bar is 1 mm.



Supplemental Figure IV-13 - Gametophores of *Ppkai2L* **mutants in red light.** Gametophore height of *Ppkai2L* mutants, compared to that of WT, *Ppccd8* and *Ppmax2-1* mutants, following 2 months growth under red light. Mutant genotypes carry mutations as indicated in Figure 8 and Supplemental Table IV-2, with asterisks for null mutations. Box plots of n = 32-36 gametophores, grown in 3 Magenta pots, harboring between 15 and 25 leaves. Statistical groups (all genotypes comparison) are indicated by letters and were determined with a Kruskal-Wallis test followed by a Dunn *post hoc* test (p < 0.05).



Supplemental Figure IV-14 - Phenotypic response of *Ppkai2L* mutants to (-)-GR24 and (+)-GR24 application:

caulonema number in the dark. *Caulonema* numbers from *Ppkai2L* mutants following application of (A) 1 μ M (-)-GR24 (in red), (B) 0.1 μ M (+)-GR24 (in turquoise) (C,D) 0.1 μ M (-)-GR24 (in red). DMSO was applied as control treatment (ctl, dark grey) except in (A), where acetone was applied. WT and both *Ppccd8* and *Ppmax2-1* mutants were used as control genotypes. Mutant genotypes carry mutations as indicated in Figure IV-8 and Supplemental Table IV-2, with asterisks for null mutations. For each genotype, *caulonema* were counted after 2 weeks in the dark, from 24 individuals, grown in 3 different 24-well plates. Statistical groups (comparing genotypes in control conditions) are indicated by letters and were determined by a one-way ANOVA with Welch test (95% Cl). Significant differences between control and treated plants within a genotype based on one-way ANOVA with Welch test: ***, p < 0.001; **, p < 0.01; *, p < 0.5.

Supplementary table IV-1 - Oligonucleotides used in this study.

Primer name/purpose	Sequence (5'—3')			
Cloning for protein expression (Gateway recombination sites are underlined, protease cleavage sites are in italic)				
PpKAI2L-	ggggacaagtttgtacaaaaaagcaggctccctggaagtgctgtttcagggcccgATGGATGAGCTACCATCACTC			
C_attb1_HRV3C				
PpKAI2L-C_attb2	ggggaccactttgtacaagaaagctgggtctcaTCAACAAGACTCCAGATTCC			
PpKAI2L-	ggggacaagtttgtacaaaaaagcaggctccctggaagtgctgtttcagggcccgATGGAGGAAGGACCAACACT			
D_attb1_HRV3C				
PpKAI2L-D_attb2	ggggaccactttgtacaagaaagctgggtctcaTTACAGACTTCCTGCGAGGT			
PpKAI2L-	ggggacaagtttgtacaaaaaagcaggctccctggaagtgctgtttcagggcccgATGGAGGAGCCATCCTTGTT			
E_attb1_HRV3C				
PpKAI2L-E_attb2	ggggaccactttgtacaagaaagctgggtctcaTTATAGACTTCCAGCGAGGT			
PpKAI2L-	ggggacaagtttgtacaaaaaagcaggctccctggaagtgctgtttcagggcccgATGCAGTCCCACAATGTGAT			
F_attb1_HRV3C				
PpKAI2L-F_attb2	ggggaccactttgtacaagaaagctgggtctcaTCATGATGCAAAACATCGCAA			
PpKAI2L-	ggggacaagtttgtacaaaaaagcaggctccctggaagtgctgtttcagggcccgATGCCGAGCCCGTTGCTCTC			
H_attb1_HRV3C				
PpKAI2L-H_attb2	ggggaccactttgtacaagaaagctgggtctcaTCATGAGTCGATGCAGTGGAG			
PpKAI2L-	ggggacaagtttgtacaaaaaagcaggctccctggaagtgctgtttcagggcccgATGATTCCGCAATCGAGCTC			
K_attb1_HRV3C				
PpKAI2L-K_attb2	ggggaccactttgtacaagaaagctgggtctcaTCACGGCGCAAGGCAGCGGAGA			
PpKAI2L-L-	ggggacaagtttgtacaaaaaagcaggctccctggaagtgctgtttcagggcccgATGGTGGTGGTGTCCGAGTCCTTG			
Δ4/_attb1_HRV3C				
PpKAI2L-L_attb2	ggggaccactttgtacaagaaagctgggtctcaTTAATCTTCAATGCAGCGAA			
Cloning for complementatio				
AtD14_promo_attB4	ggggacaactttgtatagaaaagttgccCCTCTTGTTGGATTCTTGGC			
AtD14_promo_attB1R	ggggactgcttttttgtacaaacttgcTTTTTATGTGTTTGGGTTTGAGG			
AtD14_CDS_attB1	ggggacaagtttgtacaaaaaagcaggcttcATGAGTCAACACAACATCTT			
AtD14_CDS_attB2_ΔS	ggggaccactttgtacaagaaagetgggtcCCGAGGAAGAGCTCGCCGGA			
AtKAI2_promo_attB4	ggggacaactttgtatagaaaagttgccTTCACGACCAGTATGGTTTACTCA			
AtKAI2_promo_attB1R	ggggactgcttttttgtacaaacttgcCTCTCTAAAGAAGATTCTTCTCTGGTT			
AtKAI2_CDS_attB1	ggggacaagtttgtacaaaaaagcaggcttcATGGGTGTGGTAGAAGAAGC			
AtKAI2_CDS_attB2_ΔS	ggggaccactttgtacaagaaagctgggtcCATAGCAATGTCATTACGAAT			
PCR-Based mutagenesis of <i>PpKAI2L-H</i>				
PpKAI2L-H-L28F	GTGGTGCTGGGGCATGGCTTTGGAACCGACCAATCAG			
qPCR primers				
PpKAI2L-A_qF468	ATGGCAGTGCAGGAGTTTAG			
PpKAI2L-A_qR571	GTAACACGCTGCGCAAATC			
PpKAI2L-B_qF631	CTTGCCACATCTTGCAAAGC			

PpKAI2L-B_qR735	ATGCAGCACCTCAACAATGC
PpKAI2L-C_qF525	GGAGTTTGGTAGGACGCTATTC
PpKAI2L-C_qR632	GCACAGTCACCTTTGGTAGAA
PpKAI2L-D_qF623	CACCGTCCCTTGCCATATT
PpKAI2L-D_qR736	TCTGCAACACCTCGACAATC
PpKAI2L-E_qF667	CCTTGGTAGTCGCGGATTAT
PpKAI2L-E_qR761	GAACTGAGCTGAGGCAAATG
PpKAI2L-F_qF602	TGAAAGTGCCAGTGCATCTC
PpKAI2L-F_qR744	TCACTCAAATGCGGCAAG
PpKAI2L-K_qF589	TCTTCCAGAGCGATCTACGTTC
PpKAI2L-K_qR738	GTTCAGAACCTCCATCATCGTC
PpKAI2L-H_qF671	AATTGAAGTGGCGGAGTACC
PpKAI2L-H_qR779	ACCACCAATTCTGGACAACTC
PpKAI2L-I_qF509	TGACGACAAAGCAGTGCAAG
PpKAI2L-I_qR643	TATGGCAAGGCACTGTAACCTC
PpKAI2L-L_qF453	AGGTTGCCTTGCATCTCTTG
PpKAI2L-L_qR569	AGGTCATGCTGCTCAAATCC
PpKAI2L-J_qF393	CTCATTCTCATGGCAGCATCTC
PpKAI2L-J_qR538	CCATCGCCTTAGGTACAAAACC
PpKAI2L-G/M_qF638	GCGACCAGATATTGCCCTTA
PpKAI2L-G/M_qR743	ACTCCACTTTGCACTAGATAGC
PpKUF1LA_qF	GGAGGTGCTCATTGGAACTAAA
<i>PpKUF1LA_</i> qR	GGTGCATCCGAAGCAATATCTA
<i>Pp3c6_15020_qF</i>	CAGAACGGCTTTGTGGATTTG
<i>Pp3c6_15020_qR</i>	GTCCGAGTTGGTAGAGGTAGTA
PpAPT_qF	ACTTGCCGTGGCGAGCTAC
PpAPT_qR	CATCCTTGGAGGCCGACATC
PpACT3_qF	AGCGAGTACGATGAATCTGG
PpACT3_qR	ACACAGCAAGAGCTCAATCC

Supplementary table IV-2 - Mutants used in the study.

Mutant name	Clade(s)	Mutation effects
Ppccd8		PpCCD8: deletion Proust et al 2011
Ppmax2-1		PpMAX2: Full CDS deletion, Lopez-Obando et al 2018
a2*-b4*-c2*	A-E	STOP in PpKAI2L-A (41), -B (15) and -C (64)
c2*-d4*-e1	A-E	STOP in PpKAI2L-C (64) and $-D$ (39) PpKAI2L-E: $-(A^{99}G^{100}) + (D^{99}I^{100}R^{101})$
a1-b1-c1-d1-e2*	A-E	PpKAI2L-A: -($F^{30}G^{31}$); PpKAI2L-B: -($L^{7}LEA^{10}$); PpKAI2L-C: - ($M^{55}GAGTTD^{61}$); PpKAI2L-D: -($Q^{33}S^{34}$) +(R^{33}); STOP in PpKAI2L- E (106)
a3*-b1-c3*-d3*-e2*	A-E	STOP in PpKAI2Like-A (41), -C (64), -D (41) and -E (106); PpKAI2L-B: $-(L^7 LEA^{10})$
j1*	J,G,M	STOP in PpKAI2Like-J (48)
j1*-g2*-m2*	J,G,M	STOP in PpKAI2Like-J (48), -G (174) and –M (182)
j3-g3*_m1	J,G,M	PpKAI2Like-J: -(E ¹⁸ NPY ²¹); PpKAI2Like-G -(G ¹⁶⁴ W ¹⁸⁵); PpKAI2Like-M: -(G ¹⁶⁰ DYI ¹⁶⁴)
j7*-g1-m1	J,G,M	STOP in PpKAI2Like-J (27); PpKAI2Like-G: -(G ¹⁶⁰ DYI ¹⁶⁴); PpKAI2Like-M: -(G ¹⁶⁰ DYI ¹⁶⁴)

j6-g5*-m1	J,G,M	PpKAI2Like-J: -(L ²² KAH ²⁵); STOP in PpKAI2Like-G (166);
		$PpKAI2Like-M: -(G^{160}DYI^{164})$
j8*-g1-m5*	J,G,M	STOP in PpKAI2Like-J (44); PpKAI2Like-G: -(G ¹⁶⁰ DYI ¹⁶⁴); STOP
		in PpKAI2Like-M (163)
f2*-k2*-j5	F,K ; J,G,M	STOP in PpKAI2Like-F (31); STOP in PpKAI2L-K (241);
		$PpKAI2Like-J: -(L^{22}KAHN^{26})+(Y^{22})$
Δh	H,I,L	PpKAI2L-H: Full CDS deletion
Δh -i2*	H,I,L	PpKAI2L-H: Full CDS deletion; STOP in PpKAI2L-I (62)
1h ; 3* 11*	шп	\mathbf{D} we have $\mathbf{u} = 1$ or 0 and 1 and 1 and 1 or 0
	11,1,1	PpKAI2L-H: Full CDS deletion; PpKAI2L-I: +(M VLSA); STOP
.1 4 1 (4 .) 4 1)		III PPKAI2L-L (74)
J1*-g1-m0*-13*-12	J,G,M ; H,I,L	STOP in PpKAI2Like-J (48); PpKAI2Like-G: -(G ¹⁰⁰ DYI ¹⁰⁺);STOP
		in PpKAI2L-M: 181 PpKAI2L-I: +(M ⁵⁹ VLSA ⁶³); PpKAI2L-L: -N ⁵⁹
Δh -f1*-k1*-j4	F,K ; J,G,M ;	PpKAI2L-H: Full CDS deletion; STOP in PpKAI2Like-F (31);
	H,I,L	STOP in PpKAI2L-K (233); PpKAI2Like-J: -(L ²² KAH ²⁵)
<i>∆h-f3*-k3*-j6</i>	F,K ; J,G,M ;	PpKAI2L-H: Full CDS deletion; STOP in PpKAI2Like-F (33);
	H,I,L	STOP in PpKAI2L-K (231); PpKAI2Like-J: -(L ²² KAH ²⁵)
∆h-f4*-k4* -j2*	F,K ; J,G,M ;	PpKAI2L-H: Full CDS deletion; STOP in PpKAI2Like-F (41);
	H,I,L	STOP in PpKAI2L-K (239); STOP in PpKAI2Like-J (47)
Δh -i1*-f1*-k1*-j4-g4*-m1	F,K ; J,G,M ;	PpKAI2L-H: Full CDS deletion; STOP in PpKAI2Like-I (75); STOP
	H,I,L	in PpKAI2Like-F (31); STOP in PpKAI2L-K (233); PpKAI2Like-J:
		-(L ²² KAH ²⁵); STOP in PpKAI2Like-G (165); PpKAI2Like-M: -
		(G ¹⁶⁰ DYI ¹⁶⁴)

Supplementary table IV-3 - List of gene sequences used in this study.

Sequence ID	Genebank GI number	Phytozome ID	Splicing variants (underlined variant has been used in this study)
PsRMS3	GI:1839264		
AtD14	GI:18396732		
AtKAI2	GI:15235567		
BsRsbQ	GI:757754288		
PpKAI2L-A		Pp3c2_19340	<u>Pp3c2 19340V3.1</u> ; Pp3c2_19340V3.2
PpKAI2L-B		Pp3c14_6110	Pp3c14_6110V3.1 (5'UTR sequence); Pp3c14_6110V3.2; Pp3c14_6110V3.3
PpKAI2L-C		Pp3c25_5350	Pp3c25_5350V3.1; Pp3c25_5350V3.2; Pp3c25_5350V3.3; Pp3c25_5350V3.4; Pp3c25_5350V3.5; Pp3c25_5350V3.6
PpKAI2L-D		Pp3c6_10610	Pp3c6 10610V3.1 ; Pp3c6_10610V3.2 ; Pp3c6_10610V3.3 ; Pp3c6_10610V3.4 ; Pp3c6_10610V3.5 ; Pp3c6_10610V3.6 ; Pp3c6_10610V3.7
PpKAI2L-E		Pp3c5_16420	Pp3c5_16420V3.1 ; Pp3c5_16420V3.2 ; Pp3c5_16420V3.3 ; Pp3c5_16420V3.4 ; Pp3c5_16420V3.5 ; Pp3c5_16420V3.6 ; Pp3c5_16420V3.7 Pp3c5_16420V3.7 ;
PpKAI2L-F		Pp3c10_1460	<u>Pp3c10_1460V3.1</u> ; Pp3c10_1460V3.2
PpKAI2L-G		Pp3c4_4910	<u>Pp3c4_4910V3.1 ;</u> Pp3c4_4910V3.2
PpKAI2L-H		Pp3c3_11730	Pp3c3_11730V3.1 ; <u>Pp3c3_11730V3.2 ;</u> Pp3c3_11730V3.3

PpKAI2L-I	Pp3c12_8770	<u>Pp3c12_8770V3.1</u> ; Pp3c12_8770V3.2
PpKAI2L-J	Pp3c4_32050	<u>Pp3c4_32050V3.1</u> ; Pp3c4_32050V3.2; Pp3c4_32050V3.3
PpKAI2L-K	Pp3c1_18010	<u>Pp3c1_18010V3.1</u> ; Pp3c1_18010V3.2; Pp3c1_18010V3.3;
PpKAI2L-L	Pp3c4_19700	<u>Pp3c4_19700V3.1</u> ; Pp3c4_19700V3.2; Pp3c4_19700V3.3; Pp3c4_19700V3.4; <u>Pp3c4_19700V3.5</u> (for protein expression)
PpKAI2L-M	Pp3c26_13220	Pp3c26_13220V3.1 ; <u>Pp3c26_13220V3.2</u> ; Pp3c26_13220V3.3
PpMAX2	Pp3c17_1180	
PpCCD7	Pp3c6_21550	
PpCCD8	Pp3c6_21520	
PpKUF1LA	Pp3c2_34130	
(At) Arabidopsis thaliana ; (Bs) Bacillus subtilis ; (Ps) Pisum sativum ; (Pp) Physcomitrium patens		

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CHAPTER V – What are SMXL proteins putative molecular functions and how can it be integrated in SL and KL signaling?

V-A) ClpATPases proteins in plants and beyond

SMXL proteins display high structural similarity to a broader family of proteins called Clp-ATPases, which are present in all three parts of the living realm: Bacteria, Archea and Eukaryotes. Clp-ATPases are specific members of the AAA+-ATPases superfamily (Snider et al., 2008) which take their name from their first characterized role in casein degradation (caseinolytic protease, Clp) (Schirmer et al., 1996). Unlike what their name implies, they do not carry a protease catalytic activity, but rather enable proteolytic activity. Indeed, these proteins were first discovered in bacteria, where some of these ATPases form a multimeric complex with the unrelated serine-protease ClpP component (Katayama-Fujimura et al., 1987; Mimiaga et al., 2016). These complexes have similar organization and function as the proteasome and have hence been called "proto-proteasomes" by some authors (Snider et al., 2008; Budenholzer et al., 2017; Clarke, 1999; Ali and Baek, 2020). In such complexes, Clp-ATPases act as chaperones to recognize and, using the energy taken from ATP hydrolysis, unfold proteins that will be degraded by the complex (Schirmer et al., 1996). This similar molecular mechanism was shown to enable very diverse functions in bacteria, always related to the regulation of other proteins' stability and/or activity (Wawrzynow et al., 1996). Close homologs of these ATPases are also found in plants, where ClpP partners are also present, therefore it was early suggested that such protease complexes could also exist in plants (Liebeherr et al., 2010). However, as we shall see hereafter, not all Clp-ATPases are involved in such complexes. The function of Clp-ATPases is often brought down to the removal of protein aggregates (Singh et al., 2010).



Figure V-1 – **Domain architecture of major Clp-ATPases subtypes.** N = amino-terminal domain (also called double Clp-N domain), NBD1 = first ATPase domain, M = middle domain, NBD2 = second ATPase domain, CTP = chloroplast transit peptide. bClp stands for bacterial Clp, eClp for eukaryotic Clp and pClp for plant Clp. In the absence of such precision, all members of the subfamily have a similar architecture. Note that NBD1 usually contains two Walker B motifs. Domains are not represented true to scale.

V-B) Clp-ATPases/HSP100 classification

More precisely, SMXL fall into type I Clp-ATPases (HSP100 using yeast nomenclature), as they contain two nucleotide binding domains (NBD), contrary to type II which contain only one NBD (more similar to NBD2 from type I). The two NBD are very different in sequence and in structure, except for Walker A (or P-loop) and Walker B motifs (necessary for ATP binding and hydrolysis) (figure V-1), suggesting a functional split between the two NBD (Gottesman et al., 1990). Indeed, one NBD ensures ATP hydrolysis while the other permits oligomerization (Schirmer et al., 1996). Oligomerization is a key feature of both type I and type II Clp-ATPases and is induced by either ADP or ATP binding. As exemplified by *Escherichia coli* proteins, Clp-ATPases usually form hexameric rings, with target proteins being unraveled through the very tiny central pore of the ring (Duran et al., 2017).

Across all living organisms, Clp-ATPases can be further classified into four subtypes in both type I and type II. According to the nomenclature of Schirmer and colleagues (Schirmer et al., 1996), type I contains subtypes ClpA, found in Gram negative bacteria, ClpB which is ubiquitous, ClpC that is found in land plants, algae, cyanobacteria and Gram positive bacteria, and ClpD which is specific to land plants (figure V-I). Type II contains subtypes ClpM, N, X and Y, where ClpX is ubiquitous, ClpM is specific to mammals and protozoa, and ClpY/HslU and ClpN are found in some bacteria (Mishra and Grover, 2016; Clarke, 1999). This classification in subfamilies relies on the differences in consensus sequence of Walker A and Walker B motifs, as well as specific signature sequences in the more variable Nterminal, C-terminal and middle domains (Schirmer et al., 1996).

V-C) Clp proteolytic complexes

The first Clp proteolytic complex that was discovered, and perhaps the most described since, is ClpAP. ClpP association with ClpA changes ClpP substrate specificity (see paragraph V-A), but also enhances its catalytic efficiency in E. coli (Thompson and Maurizi, 1994; Thompson et al., 1994). ATP binding, but not its hydrolysis, is necessary for ClpAP complex formation and activity on small peptides. However, to degrade larger protein substrates, the complex needs intact ClpA ATP hydrolysis activity (Thompson et al., 1994; Thompson and Maurizi, 1994; Wawrzynow et al., 1995). ClpP can be seen as a real Heat Shock Protein (HSP) in E. coli, as its expression is highly induced by heat stress, compared to its basal level in physiological conditions (Squires et al., 1991; Squires and Squires, 1992). On the other hand, ClpA is produced at similar levels along development in bacteria, consistent with its role in housekeeping/physiological growth (Squires and Squires, 1992). Still, ClpA has a protective role against other stresses besides heat and is effectively induced by anaeroby and high density cultivation (Gottesman et al., 1990). In bacteria, ClpP can also form proteolytic complexes with the other Clp ATPases ClpC (Porankiewicz et al., 1999) and ClpX (Wickner, 1999; Horwich et al., 1999). The resulting ClpCP and ClpXP complexes are not functionally equivalent to ClpAP and are more relevant to heat stress. For some Clp complexes, adapter proteins might be needed to target specific substrates (Schmidt et al., 2009). The stoichiometry of these three Clp complexes is most often one hexameric ring of Clp-ATPases (containing the same number of adaptors if needed (Kirstein et al., 2009)) at one extremity of two stacked heptameric rings of ClpP (Duran et al., 2017; Baker and Sauer, 2012).

V-D) Bi-functionality of Clp-ATPases

Several clues lead to the view that both type I and II Clp-ATPases act as chaperones in the absence of ClpP, and help to degrade abnormal proteins in complex with ClpP (Wawrzynow et al., 1995, 1996). The switch between these two "roles" would be dependent on ClpP presence but also on the energy balance, since substrate degradation depends on ATP hydrolysis. It was also hypothesized that this functional switch depends on the motifs recognized on their protein substrates (Wawrzynow et al., 1996): the hexamer of Clp-ATPase would recognize a specific motif or structure on various partially denatured proteins which would activate the ATPase activity and hence permit local unfolding of the protein. If the protein does not contain an additional motif/structure, it would be released, and the unfolded site would spontaneously correctly refold. On the other hand, if the engaged protein contains further "signatures of abnormality", a further change in the Clp-ATPase conformation would permit the recruitment of the protease ClpP partner and the now stable complex would proceed to complete degradation of the protein substrate.

V-E) Clp chaperone complexes

Among both types Clp-ATPases, ClpB subtypes have a different operating mode, as they do not associate with proteases (Clarke, 1999). Indeed, ClpB subtypes are the only Clp-ATPases that do not contain the conserved IGF/L motif enabling interaction with ClpP (Gottesman et al., 1993; Wojtkowiaks et al., 1993) (figure V-1). Interestingly, the association in oligomers is as important for ClpB function as for the others (Duran et al., 2017), so this feature is not only crucial for protease activity of Clp complexes, but also for the chaperone activity of Clp-ATPases *per se*. Moreover, ClpB (HSP104 in *Saccharomyces cerevisiae*) interact with other chaperones such as DnaK heat-shock proteins (Hsp70) and DnaJ (Hsp40) to disaggregate protein aggregates (Duran et al., 2017). ClpB by itself has a limited disaggregation ability, which is dramatically increased by association with these co-chaperones.

In *E. coli*, ClpA and B are closely related, but unlike ClpA, ClpB is a real HSP whose gene transcription is induced by heat stress (Kitagawa et al., 1991; Squires et al., 1991). Moreover, the *clpB* mutant is hypersensitive to high temperature, suggesting ClpB has a protective role against heat stress (Squires et al., 1991). The ClpB homolog in the yeast *S. cerevisiae* is called HSP104 and is also necessary for both basal long-term thermotolerance (especially in the highly thermotolerant spores) and for induced thermotolerance (Sanchez et al., 1992). Furthermore, HSP104 accumulation grants increased tolerance to many other stresses and *HSP104* expression is induced by low glucose, ethanol, arsenite and cadmium. Therefore, HSP104 has a protective, cross-tolerance, effect against many abiotic stresses (Sanchez et al., 1992). In mammals ClpB (HSP110) have been suggested to protect rRNA transcription from heat stress, which has also been suggested for *E. coli* ClpB (Squires and Squires, 1992).

The roles of Clp-ATPases as chaperones likely explains why the phenotypes of KO *Clp* mutants are often dim, as other chaperones families can ensure survival when a single Clp is absent (Squires and Squires, 1992).

V-F) ClpATPases in plants

In plants, subtypes ClpB, C and D (type I) and ClpX (type II) are present, as well as ClpP serine proteases (table V-1). Almost all genes that encode Clp components are found in the nucleus genome, as exemplified by Arabidopsis, where only ClpP1 (ATCG00670) is encoded by the plastidial genome (Ali and Baek, 2020). Therefore, it appears that *Clp* genes, like many protein-encoding genes originating from ancestral endosymbionts, have been transferred to the nuclear genome along plants evolution (table V-1).

One of the firsts that was characterized is the plastidial ClpD/ERD1 in Arabidopsis (Kiyosue et al., 1993). While it is a homolog of *E. coli ClpB* and *S. cerevisiae HSP104*, *ERD1* expression is specifically and quickly induced only by dehydration, and not by other stresses like heat, cold, carbon deficiency or metals. Later, *ERD1* expression was found to be also induced by salt stress and dark-induced senescence and etiolation, in a partially ABA-dependent way (Nakashima et al., 1997).

It was soon established that *ERD1* has at least three homologues in Arabidopsis (Kiyosue et al., 1993). Subsequently, other proteins that could be bound by anti-HSP104 antibodies were found to be accumulated in various heat-stressed angiosperms. Accumulation of AtClpB/HSP101 transcripts occurs under heat shock conditions but is not triggered by ABA treatment or by cold or osmotic stress (Nieto-Sotelo et al., 1999; Agarwal et al., 2002). These proteins correspond to *bona fide* ClpB/HSP104 homologs and were named HSP101 proteins. Arabidopsis AtHSP101 was first characterized and was found to be able to complement the yeast *hsp104* mutant (Schirmer et al., 1994). The same restoration of yeast induced thermotolerance by plant HSP101 was observed with a soybean protein (Lee et al., 1994), and later with wheat, tobacco and rice HSP101 (Agarwal et al., 2002). AtHSP101 is cytosolic, unlike most of Arabidopsis ClpATPases (Agarwal et al., 2002), however it was afterwards shown that most plants possess several ClpB homologs with diverse subcellular localizations (Singh and Grover, 2010).

On the other hand, ClpC is the functional equivalent of bacterial ClpA in flowering plants plastids (Schirmer et al., 1994; Shanklin et al., 1995). *In vitro*, plant ClpC can interact with the bacterial ClpP and degrade the same substrates, suggesting this complex was conserved across evolution, between bacteria and plastids. Indeed, like ClpD/ERD1, ClpC proteins have a long N-terminal leader domain containing a chloroplast transit peptide (Figure V-1) (Mishra and Grover, 2016). The existence of ClpCP complexes was also supported by the observation that plants possess several ClpP homologs, most being directed to plastids. Much like ClpA and ClpP in *E. coli*, Arabidopsis ClpC and ClpP are expressed in all tissues in basal conditions. Unlike in *E. coli* however, neither ClpC nor ClpP are HSP. ClpCP complexes in plastids could permit to degrade neosynthesized proteins containing wrong amino acids or incorrectly folded, therefore preventing accumulation of potentially toxic plastidial proteins in excess. It was also suggested that ClpCP degrades functional plastidial proteins normally acting in complexes when their nucleus-encoded partners are not present. Since the Ubiquitin-Proteasome System (UPS) is not present in plastids, the ClpCP complex could play a similar role in housekeeping as bacterial ClpAP complexes. Indeed, approximately 30% of plastidial neosynthesized proteins are almost immediately degraded if their partners imported from the cytosol are not present (Shanklin et al., 1995). ClpC can associate with the internal plastidial membrane, thereby permitting recognition of misfolded proteins directly from their translocation into plastidis, possibly acting on some subunits of Rubisco and of the cytochrome b₆f photosynthetic

complex (Clarke, 1999). Highlighting the vital role of ClpCP-regulated protein turnover in photosynthetic organisms, loss of function of ClpC is lethal both in plants and cyanobacteria (Clarke, 1999).

Some plant ClpP homologs are encoded by the nuclear genome (nClpP) and others are encoded by the plastidial genome (pClpP). The function of pClpP was hypothesized to be vital, since it is one of the few genes still present in a parasitic plant plastidial genome (Squires and Squires, 1992). While pClpP proteins are retained to plastids, at least some nClpP are targeted to mitochondria (Sokolenko et al., 1998; Clarke, 1999; Porankiewicz et al., 1999). Plants ClpX are also directed to this compartment, where they form ClpXP proteolytic complexes with nClpP, with similar structure and function as bacterial ClpXP complexes (Halperin et al., 2001). Such ClpXP proteolytic complexes are found in the mitochondria of animals as well, suggesting it is an ancestral eukaryotic character (Halperin et al., 2001).

	Protein type (localization)		Arabidopsis thaliana		Physcomitrium patens
Chaperone -	ClpB (cy)	1	HSP101/AT1G74310		
	ClpB (cp)	2	AT5G15450 and AT4G14670	2	Pp3c24_9060 and Pp3c8_12320
	ClpB (mt)	1	AT2G25140		
	ClpB (?)			2	Pp3c19_4790 and Pp3c12_10120
	ClpC (cp)	2	AT5G50920 and AT3G48870	5	Pp3c16_17640, Pp3c25_7180, Pp3c3_18360, Pp3c5_23010 and Pp3c6_7360
	ClpD (cp)	1	ERD1/AT5G51070		
	ClpD (?)			2	Pp3c18_15840 and Pp3c21_5430
	ClpX (mt)	3	AT5G53350, AT5G49840 and AT1G33360	1	Pp3c1_30360
	ClpX (?)			2	Pp3c1_40090 and Pp3c17_15650
Clp	ClpP (mt)	1	AT5G23140	1	Pp3c4_31230
proteolytic complex	ClpP (cp)	5	ATCG00670, AT1G666670, AT1G02560, AT1G11750 and AT5G45390	15	Pp3c23_19030, Pp3c8_17570, Pp3c24_11660, Pp3c17_16820, Pp3c14_13060, Pp3c14_13063, Pp3c5_28650, Pp3c25_15340, Pp3c2_27590, Pp3c26_8310, Pp3c4_26850, Pp3c12_2930, Pp3c3_12310, Pp3c13_21640 and Pp3c3_12000
	ClpP (?)			4	Pp3c5_27640, Pp3c16_20770, Pp3c16_17780 and Pp3c25_4310

Table V-1 – **Comparison of Clp proteins content in** *Arabidopsis thaliana* and in *Physcomitrium patens* Subcellular localization of *P. patens* proteins are only *in silico* predictions (annotation on Phytozome and prediction by DTU.dk TargetP-2.0 server). Localization is given between parentheses: cytosol (cy), plastids (cp), mitochondria (mt), undetermined (?).

Clp proteolytic complexes acting in organelles are major players in one of the two coexisting main plant systems of bacterial origin for degrading aberrant or damaged proteins, together with the proteasome complex that is active in the cytosol and nucleus (Ali and Baek, 2020). Among systems of bacterial origin, Clp complexes are particularly important as loss of function mutants are often lethal (Ali and Baek, 2020). On the other hand, much like in bacteria, loss of function of Clp-ATPases that are not in proteolytic complexes (ClpB and ClpD) is not lethal in physiological conditions. However, their function becomes vital when the plant is subjected to stress.

Interestingly, while ClpB and ClpD gene number is low in both *Arabidopsis thaliana* and *Physcomitrium patens*, there is in the latter a noticeable expansion of the ClpC and ClpP encoding genes, but not of ClpX genes (table V-1). Moreover, this expansion is not found for other protease systems of bacterial origin such as *FtsH* genes and *Deg/HtrA* genes (respectively around 20 members and 15 members for both species). This could mean that the ClpCP system in *P. patens* chloroplasts is particularly relevant to protein monitoring and turnover in *P. patens*.



Figure V-2 – **Phylogeny of SMXL proteins relative to other type I ClpATPases.** Phylogeny analysis was performed on the Phylogeny.fr platform and comprised the following steps. Sequences were aligned with MUSCLE (v3.8.31) configured for highest accuracy (MUSCLE with default settings). After alignment, regions containing gaps (and/or poorly aligned) were removed with Gblocks (v0.91b). The phylogenetic tree was reconstructed using the maximum likelihood method implemented in the PhyML program (v3.1/3.0 aLRT). The WAG substitution model was selected assuming an estimated proportion of invariant sites (of 0.012) and 4 gamma-distributed rate categories to account for rate heterogeneity across sites. The gamma shape parameter was estimated directly from the data (gamma=1.951). Reliability for internal branch was assessed using the aLRT test (SH-Like). Graphical representation and edition of the phylogenetic tree were performed with TreeDyn (v198.3) (Dereeper et al., 2008).

V-G) SMXL proteins as ClpATPases

The first described protein of the SMXL family, D53/OsSMXL7, was found to be structurally similar to type I ClpATPases, even though the sequence is divergent (Zhou et al., 2013) (figure V-2). Indeed, considering the whole groups taken as wholes, SMXL proteins and type I Clp-ATPases are only around 20 to 30% identical in sequence (Moturu et al., 2018). Nevertheless, key structural elements such as Walker A and B motifs are conserved (figure V-3), implying that SMXL proteins could have a similar molecular function as type I ClpATPases (Moturu et al., 2018). Moreover, the turnover of most SMXL proteins is likely dependent on their ATPase activity, as the stabilizing mutation of the degron motif is actually a loss of the second NDB Walker A motif. Such degron-dependent turnover has been proven at least for Arabidopsis and rice SMAX1 (Khosla et al., 2020; Wang et al., 2020a; Zheng et al., 2020) and SMXL6/7 (Wang et al., 2015b; Soundappan et al., 2015; Liang et al., 2016; Zhou et al., 2013). Still, the most notable characteristics differentiating D53 from other Clp-ATPases are the presence of an EAR transcriptional repression motif between the two NBDs (see paragraph V-H) and D53 strictly nuclear subcellular localization (figure V-3). However, as made apparent by the limited sequence homology between plant type I Clp-ATPases and SMXL, SMXL clearly group independently from Clp-ATPases and they are also restricted to land plants (figure V-2). It is also unlikely that SMXL proteins take part in proteolytic complexes with ClpP proteases, as they lack the IGF/L interaction motif (Moturu et al., 2018). Therefore, a possible molecular role of SMXL proteins would be to ensure the proper folding, and thus activity, of target proteins. Under this hypothesis, SMXL would have a similar molecular function as ClpB and ClpD, their specific sets of targets leading to very different outcomes. Notably, the role of D53/SMXL7 on inhibiting BRC1 transcription could be understood as a stabilization of the TPL co-repressor under an active conformation (Soundappan et al., 2015). On the contrary, we could imagine that SMXL interaction with their targets could sequester the targets in nuclear speckles (see paragraph V-H), hence rendering them inactive.

V-H) The multiple roles of SMXL proteins

In angiosperms, SMXL proteins can be subdivided into four functional types (Moturu et al., 2018; Walker et al., 2019): SMAX1/SMXL2, SMXL3, SMXL45 and SMXL678 (here named according to their respective Arabidopsis homologs). As written in chapter III, SMAX1 homologs are involved in KL signaling as repressors, while SMXL678 homologs have a corresponding role in the SL signaling pathway (see figures II-2 and III-4 from chapter III). On the other hand, SMXL45 homologs (at least in Arabidopsis) have been shown to act independently of SL or KL to promote early differentiation of phloem cells (Wallner et al., 2017, 2020), whereas the role of SMXL3 homologs has not been resolved yet. SMAX1 and SMXL678 contain a conserved RGKT motif (or degron) that has been shown to be necessary for their degradation (Zhou et al., 2013; Soundappan et al., 2015; Liang et al., 2016; Khosla et al., 2020; Zheng et al., 2020). These proteins also contain the EAR (Ethylene-responsive element binding factor-Associated amphiphilic Repression) hydrophobic motif (LxLxL or [F/L]DLN), which permits interaction of SMXL with the CTLH domain of TPL/TPR (Topless/Topless Related) transcriptional corepressors (Martin-Arevalillo et al., 2017). SMXL/TPL interactions are documented in Arabidopsis, where AtSMXL6 interacts with AtTPR3 (Causier et al., 2012a), while both AtSMAX1 and AtSMXL7 interact with several AtTPLs (Soundappan et al., 2015). This interaction has also been demonstrated in rice, where OsSMXL78/D53 interacts with all three OsTPLs (Jiang et al., 2013). It is interesting to note

that SMXL from different functional subclades can interact with the same TPL, therefore these interactions do not completely explain the different effects triggered by the SL and KL pathways, as already noted by Soundappan and colleagues (Soundappan et al., 2015). Alternatively, it can be argued that most of SMXL/TPL interactions were demonstrated through *in vitro* approaches and might therefore not faithfully reflect what happens *in planta*. At least in rice, OsSMXL78/D53 also interact with IPA1/SPL14 (Song et al., 2017) (IDEAL PLANT ARCHITECTURE 1/SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 14), a transcription factor from the SPL family. This interaction enables D53 to block IPA1-induced *TB1 (TEOSINTHE BRANCHED 1,* the rice ortholog of *BRC1)* expression. As such, IPA1 is hence a positive actor in the rice SL signaling pathway, downstream of D53. Furthermore, IPA1 can induce the expression of *D53* itself, thereby mediating SL-triggered *D53* increase in expression (Song et al., 2017). This mechanism seems a conserved feature of monocots, as it was also demonstrated in bread wheat (Liu et al., 2017). However, in dicots, SPL transcription factors and the SL pathway seem to operate in parallel in the control of axillary branching, at least in Arabidopsis (Wang et al., 2020b) (Figure V-3). Nevertheless, the EAR motif is probably not necessary for all roles of SMXL proteins, as was shown for AtSMXL7 (Liang et al., 2016).



Figure V-3 – Scheme of the different feedback regulation mechanisms of SL-associated *SMXL* genes expression in Arabidopsis and rice. Homologs are shown as identical symbols: SMXL6 (Arabidopsis) \approx D53 (rice, *Oryza sativa*), SPL9/15 \approx IPA1, BRC1 \approx TB1. Adapted from (Song et al., 2017; Wang et al., 2020b).

SMXL are strictly nuclear-localized proteins (Yang et al., 2015; Wallner et al., 2017; Zhou et al., 2013), similarly to MAX2 (Zhou et al., 2013; Shen et al., 2007), while D14 (Liang et al., 2016; Zhou et al., 2013) and KAI2 (Lee et al., 2018) can be found in both the nucleus and the cytosol. D14 can even be found outside of cells, as evidenced by its transport through the phloem in rice and in pea (Kameoka et al., 2016). SMXL7 and SMAX1 have been shown to localize more specifically to speckles inside the nucleus (Soundappan et al., 2015; Liang et al., 2016), as well as D53 (Zhou et al., 2013), but the identity and functional relevance of these structures have not been further investigated. D14 is apparently recruited to these speckles upon SL signaling (like TPR2), while SL-induced interaction between D14 and MAX2 occurs across the whole nucleus (Liang et al., 2016). These speckles might reveal SMXL7 association with active transcription sites where its repressive activity is needed. Liang and colleagues also reported that SMXL6 and SMXL7 in Arabidopsis can interact together, hinting at the formation of oligomeric structures, reminiscent of other Clp-ATPases characteristics (Liang et al., 2016) (see previous paragraphs V-C and V-E).

The first studies investigating SMXL proteins were published in 2013 (Zhou et al., 2013; Jiang et al., 2013; Stanga et al., 2013). However *smxl* mutations had been evidenced much earlier in 1977, when Iwata and colleagues obtained the *d53* (*Ossmxl7*) gain of function mutation in rice, in a forward genetics mutagenesis screen for dwarf mutants (Iwata, 1977). When this same mutant was extensively characterized in 2013, it was discovered to encode a stabilized version of the D53 protein (where the RGKT degron motif is deleted), leading to several phenotypic defects, notably hyper branching (Zhou et al., 2013). This mutant was then linked to SL since *d53* overexpresses *D10/OsCCD8* and overproduces SL, while it has a decreased *FC1/OsBRC1* expression, suggesting SL signaling is hindered. The WT D53 protein is degraded by the proteasome in response to SL in a D3/OsMAX2 and D14 dependent fashion, while the mutant d53 protein remains stable. As a RNAi knock down of D53 expression in *d3* and *d14* backgrounds restores almost completely the hyper branching phenotype, it seems that D53 is the major target (if not the only target) of D3 in rice. The same year (2013), the *smax1* loss of function mutation was identified in Arabidopsis, in an suppressor screen of *max2* elongated hypocotyl and delayed germination phenotypes (Stanga et al., 2013). However, a similar KAR/KL-induced degradation of SMAX1 was only experimentally evidenced this year, in rice (Paszkowski et al., 2020; Zheng et al., 2020) and in Arabidopsis (Khosla et al., 2020). The association of KAI2, MAX2 and SMAX1/SMXL2, in a KAR/KL-dependent way, was also demonstrated recently in Arabidopsis (Wang et al., 2020a) (chapter III).



Figure V-4 – **Domain structure and motifs of a typical SMXL protein.** Domain names written in blue are SMXL-specific notations. The N-terminal double ClpN domain and approximately the first half of the second NBD (NTPase 1 subdomain) are highly conserved between SMXL proteins, as well as with other type I Clp-ATPases. The first NBD is variable, and its Walker motifs are often difficult to spot (as in figure V-1, Walker B motifs are shown by red bars). The NBD2 is elongated in C-terminal relative to other Clp-ATPases (NTPase 2 subdomain) but is not highly conserved across SMXL proteins. The M domain is the most versatile, including amongst SMXL proteins, together with the spacer sequence between the two subdomains of NBD2, which nevertheless contains the conserved EAR motif. Highly variable regions are shown as pale boxes with dotted outlines, conserved regions are depicted as vivid colored boxes with continuous outlines.

V-I) SMXL proteins phylogeny

The SMXL family evolutionary history appears quite complicated. Two recent studies on this subject came to the conclusions that SMXL genes are exclusively found in land plants and that they extensively diversified in the angiosperm lineage (Moturu et al., 2018; Walker et al., 2019). Outside of seed plants, only one type of SMXL is found, which is most similar to angiosperms and gymnosperms SMAX1 (aSMAX1 and gSMAX1). One notable exception to that is the presence of a second SMXL type in bryopsids mosses (Walker et al., 2019) (that is, most of mosses), probably as the result of a Whole Genome Duplication (WGD) event (Bythell-Douglas et al., 2017) (see figure V-6). Gymnosperms only possess two clades: gSMAX1 and gSMXL4 (Walker et al., 2019). gSMAX1 and gSMXL4 do not broadly differ from non-seed plants sequences and from one another, therefore they did not diverge a lot from the ancestral SMXL. On the contrary, SMXL are much more diversified in angiosperms, in which they fall into 4 clades corresponding to their demonstrated functions in Arabidopsis (Walker et al., 2019; Moturu et al., 2018) (figure V-5). Interestingly, phylogenetical classification of angiosperms SMXLs is also reflected by their gene expression profiles in Arabidopsis (Moturu et al., 2018). As a matter of facts, AtSMAX1 is broadly expressed at similar (high) level except in roots, AtSMXL3 is mostly expressed in roots, AtSMXL7 is mainly expressed in axillary branches, and AtSMXL4/5 are lowly expressed everywhere and associated with the vasculature (Stanga et al., 2013; Wallner et al., 2017; Yang et al., 2015). It is puzzling that all Arabidopsis SMXL genes seem to be expressed in vascular tissues (Soundappan et al., 2015; Wallner et al., 2017).

The following evolutionary scenario is predicted (Walker et al., 2019): SMAX1 and SMXL4 superclades present in the seed plants common ancestor were duplicated in the angiosperms lineage: SMAX1 gave rise to aSMAX1 and aSMXL78, while aSMXL4 and aSMXL39 arose from SMXL4. Further duplications in dicots produced SMXL7 and SMXL8, and SMXL3 and SMXL9. Emergence of SMXL2 from aSMAX1 duplication and SMXL5 from aSMXL4, as well as the loss of SMXL9, are specificities of the *Brassicaceae* (Walker et al., 2019). These increases in SMXL family size correlate well with known WGD events, notably the ζ WGD predating the split between gymnosperms and angiosperms, as well as the ϵ WGD at the base of the angiosperms lineage (Clark and Donoghue, 2018) (see figure V-6).



Figure V-5 – **Phylogeny of SMXL proteins.** Adapted from (Walker et al., 2019). Angiosperms SMXL clades are named following Walker et al., 2019 (red) and Moturu et al., 2018 (blue).



Figure V-6 – **Global view of SMXL proteins evolutionary history.** The number of functional SMXL subtypes are indicated at the bottom and color-coded. WGD = Whole Genome Duplication.

SMXL origin at the base of land plants, together with their structural similarity to ClpATPases, suggest that these proteins are indeed derived from a ClpATPase ancestor. The question as to why such highly neofunctionalized proteins were evolutionary conserved and then recruited in diverse processes including response pathways to two phytohormones (chapter III) is very intriguing.

Loss of the degron is specific to aSMXL39 and aSMXL4 (Moturu et al., 2018; Walker et al., 2019). aSMXL39 and aSMXL4 are probably neofunctional respective to the ancestral seed plants SMXL4, because loss of the degron implies both increased stability and loss of the interaction with KAI2/D14. On another note, the EAR motif is very conserved in aSMXL39 and aSMXL4, thus they probably retain a transcriptional role (Moturu et al., 2018). Moreover, they likely play similar molecular functions as other SMXL clades, since expression of *AtSMAX1* under the *AtSMXL5* promoter restores *Atsmxl5* mutant phenotypes (Wallner et al., 2017).

The degron (RGKT) and EAR motifs are probably ancestral features, the degron being probably responsible for interaction with proteins from the KAI2/D14 family (figure V-4) (Shabek et al., 2018; Struk et al., 2018). The ancestral SMXL was probably closer to aSMAX1 and gSMAX1 and was likely involved in KL signaling and degraded in a MAX2 dependent fashion (see chapter III). This function is also currently assumed for the lone SMXL homolog of non-seed plants (Walker et al., 2019). Alternatively, gSMAX1 and non-seed plants SMXL proteins might be common, bifunctional targets of KL and SL signaling.

In order to settle between these two main hypotheses on SMXL function in mosses, we used *P. patens* as a model (chapter VI).

Chapter VI - The moss *Physcomitrium patens* SMXL homologs are negative regulators of growth acting downstream of PpMAX2

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This chapter is presented as a research paper that will be submitted, in a less detailed form, to The Plant Cell. The method chapter we refer to in methods of the present paper is provided as an annex to the thesis manuscript and is to be published in the Methods in Molecular Biology book series.

Authors contributions:

A.G is the main author of the manuscript and performed most of the experiments. Some steps in genotyping mutant and transgenic lines and support with molecular cloning was given by P.L.B, and Y2H experiments were done by S.S. Plasmids used for Ppsmxl mutants generation in the first strategy, as well as higher order Ppsmxl mutants were obtained by M.L.O. Preparation of protein extracts for GFP-trap pull-down was done with the kind help of A.J. Acquisition of GFP-trap pull-down data was done by the VIB proteomics platform in Ghent, and MS/MS data analysis was carried out by D.C. S.B and M.L.O supervised the project. S.G and S.L collaborated in the investigation of PpSMXL interactions. S.B, A.S.G and C.R contributed to experimental design and to the argumentation developed herein.

Short title

SMXL are negative regulators of the MAX2-dependent pathway in Physcomitrium patens

Abstract

SMXL proteins are a plant specific clade of HSP100/Clp-ATPases from type I (with two ATPase domains). SMXLs encoding genes have been identified in virtually all land plants' genomes but these genes have been characterized in only a small subset of land plants, e.g. *Arabidopsis thaliana* and *Oryza sativa*. In *A. thaliana*, three SMXL subclades with different functions have been identified: SMAX1/SMXL2, SMXL345 and SMXL678. Out of these, two subclades ensure transduction on endogenous signals e.g. hormones: SMAX1/SMXL2 are involved in KAI2-ligand signaling (KL, mimicked by karrikins (KAR)), while SMXL678 are involved in strigolactones (SL) signaling. Many questions remain regarding the precise cellular and molecular function of these proteins, as well as regarding their ancestral role. To address this second question, we investigated the function of the 4 *SMXL* genes found in the genome

of the Bryophyte *Physcomitrium patens*. We demonstrate that PpSMXL proteins are negative regulators of growth playing in the likely ancestral MAX2-dependent KL signaling pathway. Moreover, two of these proteins also function as positive regulators of *P. patens* SL signaling pathway.

Introduction

Strigolactones are recently characterized phytohormones with an early origin in land plants evolution. These molecules have first been identified as rhizospheric signals with both negative and positive outcomes for the producing plant: strigolactones stimulate parasitic plants seed germination (Cook et al 1966) but also promote AM symbiosis (Akiyama et al.) by boosting AM fungi mitochondrial metabolism and thus hyphae growth. Strigolactones are also employed as phytohormones in Angiosperms, where they have been linked to diverse roles in regulating plant architecture, in response to nutrient availability (see Waters et al., 2017 for a recent review). One of the most extensively studied roles of strigolactones is their ability to repress axillary branching, by inhibiting axillary bud activity, denoting them as the sought-after second player in the apical dominance theory. Strigolactones involvement in mycorrhization and probably later in plant architecture imply that these molecules played major roles in land plants evolution, for land colonization and then for the emergence of Angiosperms. However, strigolactones roles and associated cellular pathways in early-diverging plants are pretty much unknown, whilst this knowledge is necessary to understand how strigolactones could have contributed to land colonization. Still, it has been previously reported that strigolactones repress filaments branching and elongation (Proust et al., 2011; Hoffmann et al., 2014) and enhance resistance to phytopathogenic fungi (Decker et al., 2017) in the moss *Physcomitrium patens* (*P. patens*), showing this moss possesses a functional strigolactones signaling pathway. Phylogenetic studies suggest that strigolactones' biosynthesis pathway could be ancient, genes encoding most SL biosynthesis hormones being found both in land plants and some charales (Delaux et al., 2012). Notably, it has been demonstrated that the CCD8 enzyme has the same role in *P. patens* as in the Angiosperm pea (Proust et al., 2011; Decker et al., 2017). But the conservation is more contrasted when considering the signaling pathway genes. As a matter of facts, it has been shown that the *P. patens* homolog of MAX2, a F-box protein that plays a key role in strigolactones signaling, is not necessary for response to strigolactones but is rather involved in the response to red light and possibly in KAI2-Ligand (KL) signaling (Lopez-Obando et al., 2018, chapter IV). This might extend to role of the ancestral MAX2 protein. SMXL678/D53 proteins, which are type I Clp-ATPases described as repressors of strigolactones signaling in Angiosperms (Soundappan et al., 2015; Wang et al., 2015b), also have homologs in *P. patens*. In the former, these repressors must be degraded by the 26S proteasome, via the action of an SCF complex containing MAX2, for responses to SL to occur. Their degradation relies on their having a specific degron motif (RGKT) and their activity as repressors partly relies on an EAR motif mediating transcriptional repression. Angiosperms possess two other subclades of SMXL proteins: the SMAX1/SMXL2 subclade is involved in the transduction of the KL signal in a MAX2-dependent pathway, while the SMXL345 subclade is involved in cell differentiation (Moturu et al., 2018; Walker et al., 2019). Here, we show that PpSMXL proteins of *P. patens* possess a similar structure as subclades SMXL678 and SMAX1/SMXL2 proteins, with a conserved EAR motif and similar domain organization. Despite only two of them carrying a conserved degron motif, all four homologs are involved in the same pathway as PpMAX2 where they play a negative role downstream of PpMAX2. Moreover, two out of the four PpSMXL proteins could act as positive actor of SL signaling and thus constitute a level of crosstalk between this pathway and the PpMAX2-dependent pathway. The finding that none of these SMXL homologs are repressors in the SL signaling pathway supports the hypothesis that SL sensitivity has an independent origin in moss and seed plants, even though the same family of receptors was recruited in both lineages (Bythell-Douglas et al., 2017, chapter IV).



Figure VI-1 – **Model of** *PpSMXL* **genes.** Genomic sequences of the four PpSMXL genes were recovered from Phytozome (V3.6 of *P. patens* genome). Architecture of the primary (V3.1) transcript is shown for each. Only exons and inter-exonic introns are represented true to scale. Exons are shown as grey boxes, inter-exonic introns as black angles, UTRs as solid black lines and introns located in the 5'UTR are depicted as blue angles. Guide RNAs used for mutagenesis are depicted as black triangles (they are named according to their location relative to the ATG and according to their orientation), see supplementary table for their sequences. Degron (RGK/RT) and EAR motifs are shown by red and purple small boxes, respectively. Positions are indicated relative to the START codon.

Results

Phylogeny and expression analysis reveal two clades of SMXL genes in P. patens

We recovered four SMXL genes from *Physcomitrium patens* genome (V3.6, formerly *Physcomitrella patens*) on Phytozome as the first four results of a BLAST against P. patens proteome using the full protein sequence of AtSMAX1 (encoded by AT5G57710.1). The predicted PpSMXL proteins contain the four regions characteristic of the SMXL family: a double ClpN N-terminal domain (N) and two ATPase domains (D1 and D2) separated by a long middle domain (M) (Supplemental Figure VI-1). They correspond to the same proteins reported in a recent phylogenetic study (Walker et al., 2019). As per this study, proteins encoded by *Pp3c2* 14220 and *Pp3c1* 23530 (here respectively renamed as *PpSMXLA* and *PpSMXLB*) correspond to a SMXL clade that is specific of bryopsid mosses, while those encoded by Pp3c9_16100 and Pp3c15_16120 (PpSMXLC and PpSMXLD herein) correspond to a SMXL clade that is common to all mosses. Concordantly, this organization is evidenced by these genes' structure, with *PpSMXLA* and *PpSMXLB* possessing an extra exon when compared to *PpSMXLC* and *PpSMXLD* (Figure VI-1). This split is also noticeable at the protein level, as PpSMXLC and PpSMXLD are identical at 72% and PpSMXLA and PpSMXLB are identical at 61%, whereas other comparisons give 27-29% identity. This shows that these two PpSMXL clades have evolved separately for a while. To further explore the putative divergence between these two clades, we assessed expression of the four *PpSMXL* genes, firstly by staining two-week-old *proPpSMXL:GUS* lines. We found that *PpSMXLA* promoter is hardly active at this age, as staining was only observed occasionally at the tip of phyllids (Figure VI-2A, Supplemental Figure VI-2). *PpSMXLC* and *PpSMXLD* promoters seem to be much more active since GUS staining was noted in most tissues (Figure VI-2A, Supplemental Figure VI-2). On the other hand, the age of the plant had little effect for *PpSMXLA*. We could not obtain proPpSMXLB:GUS lines, as the PpSMXLB promoter region could not be amplified by PCR. Nevertheless, using these GUS lines, we confirmed the difference of expression levels given by the *P. patens* eFP-Browser (Supplemental Figure VI-3, original data from Ortiz-Ramírez et al., 2016). We then investigated these genes' expression along vegetative development, using RT qPCR on samples from different organs: protonema at 6 days (mostly chloronema in our culture conditions), at 10 days (mix of chloronema and caulonema) and at 14 days (mix of chloronema, caulonema and buds), and mature gametophores and rhizoids at 35 days. Using this method, we could determine *PpSMXLB* level of expression and found that it is higher than that of *PpSMXLA* and lower than that of *PpSMXLC* and *PpSMXLD* (Figure VI-2B). We could not distinguish any significant expression differences across protonema development, for either of the four genes. However, *PpSMXL* genes all tended to be more expressed later, in mature gametophores and/or rhizoids (Figure VI-2B). Taken together, these analyses show another divergence between the two PpSMXL clades, with A/B being lowly expressed and C/D being expressed at an average level (compared to the mean expression level of P. patens genes which is around 16 RPKM, PpSMXLC/D levels are approximately 18 RPKM and *PpSMXLA/B* levels are around 2 and 6, respectively (Perroud et al., 2018)).



Figure VI-2 –**Expression of** *PpSMXL* genes along *P. patens* vegetative development. (A) GUS staining of 2-week old *proPpSMXL:GUS* plants. Scale bars are 500 μ m. (B) Transcript levels of the four *PpSMXL* genes, relative to the two reference genes *PpACT3* (*Pp3c10_17080*) and *PpAPT* (*Pp3c8_16590*). RT-qPCR data used for the analysis was extracted from 4 biological replicates, each with two technical repeats. Point represents the mean of two technical repeats. Some points were excluded from analysis following an outliers identification test carried out in GraphPad Prism (version 8.4.2). Statistical significance scores (*p*<0.05) of comparisons among tissues are indicated as bold letters (Kruskal-Wallis test followed by a Dunn *post-hoc* test). Note the differences in expression value on y axes.

Expression of *PpSMXL* genes is slightly affected by (±)-GR24 enantiomers but very sensitive to light

In Angiosperms, AtSMXL7 and OsD53 are upregulated in response to the synthetic compound (\pm) -GR24 treatment. (±)-GR24 is known to stimulate both the SL and the KAI2-dependent pathway (putative KL pathway) in Angiosperms, as the enantiomers contained in this racemic mix have different target pathways: (+)-GR24 stimulates the SL pathway, (-)-GR24 stimulates both pathways (Scaffidi et al., 2014; Wang et al., 2020a). Hence, we tested separately the effect of the two (\pm)-GR24 enantiomers on *PpSMXL* genes expression to check if these genes are transcriptionally regulated through the SL and putative KL pathways. We assessed this response in the dark and in control light conditions (long day regimen), as we found previously that light could modify the effect of these molecules on transcript levels of putative SL and KL-related genes (Lopez-Obando et al., 2016a). In dark conditions, we found that *PpSMXLA* is slightly induced by (+)-GR24 but not impacted by (-)-GR24 (Figure VI-3, grey panels). On the opposite, *PpSMXLC* transcript levels are mildly increased by (-)-GR24 but not impacted by (+)-GR24. PpSMXLB behaves as PpSMXLA, although its induction by (+)-GR24 is even higher. On the other hand, *PpSMXLD* does not appear to be regulated by either enantiomer in the dark. In the light, the response to (-)-GR24 was abolished for *PpSMXLC*, similarly to the response to (+)-GR24 of *PpSMXLA*, which even tended to become inverted (Figure VI-3, white panels). Again, *PpSMXLB* followed the same trend as *PpSMXLA*. On the contrary, *PpSMXLD* was upregulated by (-)-GR24 in the light. These moderate effects of (±)-GR24 enantiomers are in the same amplitude range as those reported for Angiosperms SMXL genes Finally, we noted that all four genes were significantly more expressed in the dark than in the light: If we compare levels of expression in control samples, *PpSMXLA* is decreased more than 10 fold, *PpSMXLB* approximately 4 fold, *PpSMXLC* almost 7 fold and *PpSMXLD* around 4 fold in plants grown in a control light regimen compared to plants kept in the dark for one week (Figure VI-3). Taken together, these results demonstrate that (1) light signals likely repress *PpSMXL* expression; (2) PpSMXLA and PpSMXLB could be involved in the response to (+)-GR24; (3) PpSMXLC and PpSMXLD could be involved in the response to (-)-GR24, with predominance of PpSMXLD effect over PpSMXLC in the light; (4) light signals highjack *PpSMXLA*, *PpSMXLB* and *PpSMXLC* transcriptional response to GR24 enantiomers.

PpSMXL genes expression is not widely impacted by PpCCD8-derived signals but is dependent on the PpMAX2dependent pathway

In order to determine whether endogenous compounds produced via PpCCD8 activity (probably carlactone and derived non-canonical SL) have equivalent effects as (+)-GR24 on *PpSMXL* expression, we replicated the previous experiment in *Ppccd8* mutant plants (Proust et al., 2011). In dark conditions, all four *PpSMXL* genes had similar response profiles to (+)-GR24 and (-)-GR24 in *Ppccd8* as in WT and their expression levels were little affected by the loss of *PpCCD8* function. Thus, endogenous SL-like compounds produced via PpCCD8 enzymatic activity have little effect on *PpSMXL* expression in the dark (Supplemental Figure VI-4, grey panels). In the light, there was virtually no change again for *PpSMXLA*, *PpSMXLB* and *PpSMXLC*. However, *PpSMXLD* control levels were increased and the gene became unresponsive to (-)-GR24, suggesting that PpCCD8-derived compounds inhibit *PpSMXLD* expression and that these compounds are somehow necessary for (-)-GR24-mediated *PpSMXLD* induction (Supplemental Figure VI-4, white panels). Taken as a whole, *PpSMXL* expression analysis in the *Ppccd8* mutant reveals no significant effect of endogenous PpCCD8-derived compounds, except on PpSMXLD in light conditions. Treatments with (\pm) -GR24 enantiomers were also carried out in the *Ppmax2-1* loss of function mutant (Lopez-Obando et al., 2018), to investigate the role of *PpMAX2* in *PpSMXL* transcriptional regulation. In dark conditions (Supplemental Figure VI-4, grey panels), all four PpSMXL genes tended to be less expressed in the Ppmax2-1 mutant than in WT and this difference was significant for *PpSMXLA* and *PpSMXLC*. Furthermore, *PpSMXLA* transcript level was not induced by (+)-GR24 anymore, while it became significantly repressed by (-)-GR24, reinforcing a trend that was already seen in WT. Likewise, *PpSMXLB* expression profile changed in a comparable manner. On the contrary, *PpSMXLC* became responsive (increase) to (+)-GR24 and ceased to respond to (-)-GR24, the same trend of inversion was observed for *PpSMXLD* (Supplemental Figure VI-4, grey panels). Hence, in dark conditions, *PpMAX2* likely induces *PpSMXL* expression in the absence of exogenous compounds, while it is needed to regulate response to (\pm) -GR24 enantiomers, in an opposite way for the *PpSMXLA/B* and the *PpSMXLC/D* clades. In light conditions (Supplemental Figure VI-4, white panels), PpMAX2 loss of function resulted in a switch towards dark conditions' behavior for PpSMXLA and *PpSMXLB*, with *PpSMXLB* even being repressed by (-)-GR24 treatment. On the other hand, *PpSMXLC* and *PpSMXLD* became responsive to (+)-GR24 (increase) (Supplemental Figure VI-4, white panels). Therefore, in the light also, lack of PpMAX2 results in a switch of responsiveness to (±)-GR24 enantiomers. Considered together, these results show that *PpSMXL* genes' transcription is mainly regulated by light and responds in opposite trends to (\pm) -GR24 enantiomers, both regulations being permitted at least partly via PpMAX2, with again a split of behavior between the two PpSMXL clades. It is also important to note that, at least regarding *PpSMXL* expression, the *Ppmax2-1* loss of function mutant remains responsive to (-)-GR24, further confirming our previous conclusion that (-)-GR24 is not a perfect mimic of the signal perceived through PpMAX2 (likely KL) (see Chapter IV).



Figure VI-3 – **Expression of** *PpSMXL* genes in response to (±)-GR24 enantiomers, in the light and in the dark. Transcript levels of the four *PpSMXL* genes, relative to the two reference genes *PpElig2* (*Pp3c14_21480*) and *PpAPT* (*Pp3c8_16590*). Two-week-old plants were incubated in the dark for one week and then treated with 1μ M (+)-GR24 (diagonal hatches) or (-)-GR24 (horizontal hatches) or DMSO (control, solid color) for 6 hours, also in the dark (grey background). The same experiment was repeated in white light (white background). For each treatment, six biological replicates and two technical replicates were used. Points are the mean of the two technical replicates, coloured bars represent medians. 2-fold differences in median values are estimated as significant (DE).

Expression of *PpSMXL* genes is regulated by light in a complex way

Wanting to clarify the effect of light on *PpSMXL* expression, we checked whether this expression was also impacted by shorter exposure times and specifically by red light, as was shown for *PpMAX2* (Lopez-Obando et al., 2018). First, to assess the validity of our experimental setup, we measured the expression of known light responsive genes PpPOR1 and PpHY5a. These two control genes were elevated by red light in the medium and short term (respectively), showing that our light treatments worked (Figure VI-4). Examining *PpSMXL* response to these light conditions, we found first that all four genes indeed tend to be repressed by white light in WT plants (Figure VI-4). The amplitude of white light effect was dimmer than noted before, consistently with the shorter time of exposure (24 hours here, instead of one week of long days white light regimen in Figure VI-3). Unexpectedly, continuous red light for 24h did not have the same effect as white light for *PpSMXLA* and *PpSMXLC*, suggesting blue light might also play an important role in regulating these two genes expression. On shorter term, all except *PpSMXLD* were induced transiently by red light, the most early responsive one being *PpSMXLC*. In the absence of PpCCD8 (*Ppccd8* mutant, Supplemental Figure VI- 5), all except *PpSMXLD* became more responsive to red light (*PpSMXLA* even became responsive earlier, at 1h), suggesting PpCCD8-derived compounds might have an inhibitory effect on these genes' transient induction by red light. In *Ppmax2-1*, the light response profile of *PpSMXLA* and *PpSMXLB* did not change, whereas *PpSMXLC* and *PpSMXLD* somehow became less responsive to red light (Supplemental Figure VI-5). Moreover, *PpSMXLD* expression in dark conditions was here lower in the *Ppmax2-1* mutant than in WT, confirming the positive effect of *PpMAX2* on *PpSMXLD* expression in the dark. Hence, the response of *PpSMXL* genes to red light seems to be highly dynamic and the early regulation by red light of *PpSMXLC* and *PpSMXLD* seems to rely on *PpMAX2*. Moreover, *PpSMXLA* and *PpSMXLC*, in their respective clades, appear to be the main targets of this regulation by light.


Figure VI-4 – **Expression of** *PpSMXL* genes in response to light. Transcript levels of the four *PpSMXL* genes, relative to the two reference genes *PpElig2 (Pp3c14_21480)* and *PpAPT (Pp3c8_16590)*. Two-week-old plants were transferred in the dark for 5 days and then either left in the dark for another 24h, put in red light (RL) for 1, 6 or 24 hours, or in white light (WL) for 24 hours. For each treatment, six biological replicates and two technical replicates were used. Points are the mean of the two technical replicates, coloured bars represent medians. 2-fold differences in median values are estimated as significant (DE). *PpPOR1 (Pp3c12_20650)* and *PpHY5a (Pp3c7_11360)* profiles are given as controls of light effects. Some points were excluded from analysis following an outliers identification test carried out in GraphPad Prism (version 8.4.2).

PpSMXL proteins are localized mainly in the nucleus

Functional nuclear localization signals (NLS) were found in AtSMXL7 (Liang et al., 2016) and OsSMAX1 proteins (Choi et al., 2020). This N-terminal region is broadly conserved in the four PpSMXL proteins, suggesting they are also located in the nucleus (Supplemental Figure VI-6A). Such localization was also supported by in silico predictions based on the protein sequences, except for PpSMXLD (Supplemental Figure VI-6 B and C), although the predicted NLS have a C-terminal localization. Using stable P. patens transgenic lines overexpressing PpSMXL with a N-terminal GFP tag under the maize ubiquitin promoter (*pZmUbi:GFP-PpSMXL* lines), we could confirm the nuclear localization of *PpSMXLA*, *PpSMXLC* and *PpSMXLD*, even though the GFP signal was apparently not restricted to this compartment in several lines (Figure VI-5). To further support this finding, we then transiently overexpressed RFP-PpSMXL fusion proteins under the 35S promoter in *Nicotiana benthamiana* epidermal cells stably expressing CFP tagged H2b histone (thus nuclei were marked by CFP, Supplemental Figure VI-7A). This second method also revealed PpSMXL to be nuclear, even though overexpression under the 35S promoter, together with the use of the p19 silencing inhibitor, could be responsible for the observed leakage of the RFP signal in the cytosol (Supplemental Figure VI-7Aa-c-e-g). An alternative explanation for this cytosolic RFP signal would be that PpSMXL are actually nucleo-cytosolic, as suggested by the observation of some pZmUbi:GFP-PpSMXL lines (Figure VI-5). Nevertheless, a nuclear localization, even partial, taken together with the conservation of the EAR motif in all four PpSMXL (Figure VI-1), could point to a role in the regulation of genes' expression, similarly to SMXL homologs in Angiosperms.



Figure VI-5 – Subcellular localization of GFP-PpSMXL fusion proteins in *protonema* of transgenic *proZmUbi:GFP-PpSMXL P. patens* lines. Scale bars are 50µm. Arrow points to a nucleus.

PpSMXL proteins are not degraded rapidly in response to (+)-GR24

Since the RGKT degron motif responsible for SL triggered SMXL proteasomal degradation is found in PpSMXLC and PpSMXLD proteins (it is changed to RGRT in PpSMXLA and PpSMXLB, Supplemental Figure VI-7, B) we tried to assess whether (+)-GR24 treatment could affect these proteins levels and/or localization. We could not see any effect of a 20 minute-long 1 μ M (+)-GR24 treatment on *pZmUbi:GFP-PpSMXLA* and *pZmUbi:GFP-PpSMXLC* lines (Supplemental Figure VI-8). Similarly, *N. benthamiana* plants transiently expressing *p35S:RFP-PpSMXL* constructs did not reveal any clear modification of the RFP signal intensity or localization after a 20 minute-long 5 μ M (+)-GR24 treatment (Supplemental Figure VI-9). Moreover, in the absence of (+)-GR24 treatment, we found similar levels of RFP-PpSMXL fusion proteins whether the degron motif was present or deleted (Δ RGRT and Δ RGKT lines, Supplemental Figure VI-7A), when the fluorescent signal is typically enhanced with degron-less SMXL proteins in Angiosperms.

PpSMXLA - Pp3c2_14220 WΤ 310 TEQERHHSLSQRIQELRRKWQFVCSNSHSERCITMDSSPARDRGLGPPSHNWMKASILNN 369 310 TEOERHHSVKENTRAPS 326 a1 310 TEQERHHFHREYKSSVVNGNLFAVTHTQKDASLWTLRQLEIGDLVRHLITG 360 a2 310 TEQERHHFTENTRAPS a3 a4 310 TEQERHHSL 318 a10 310 TEQERHHSAFTENTRAPS 327 WΤ 8 VQNTLSLPAQQVLRQAI SAARERGHAQVQPLHVAFVLLAHGDPVLRQACADTHSQTLHGLHQCHA 72 8 VQNTLSLPAQQVLRQAISAARERGHAQVQPLHVAFVLLAHGDPVPTPGMC 57 a5 a6 8 VQNTLSLPAQQVLRQAISAARERGHAQVQPLHVAFVLLAHGDPVLIRILIPCTGCINATL 67 a7 8 VQNTLSLPAQQVLRQAISAARERGHAQVQPLHVAFVLLAHGDPVSHVLIRILRPCTGCINATL 70 **a8** 8 VQNTLSLPAQQVLRQAISAARERGHAQVQPLHVAFVLLAHGDPVPGMC 55 a9 8 VQNTLSLPAQQVLRQAISAARERGHAQVQPLHVAFVLLAHGDPGMC 53 all 8 VQNTLSLPAQQVLRQAISAARERGHAQVQPLHVAFVLLAHGEPGMC 53 a12 8 VQNTLSLPAQQVLRQAISAARERGHAQVQPLHVAFVLPGMC 48 PpSMXLB-Pp3c1 23530 WT 330 LHSQQLYRKWLLTCMTSHPERQVNSVSSPGRDGGPSLLHHSWINASIPNNAENSGLVIPKS 390 b1 330 LHSQQLYRKWLLTCMTSHLSIQFVTIQKASQFSFFAR 366 b2 330 LHSQQLYRKWLLTCMTS 346 b3 330 LHSQQLYRKWLLTCMTSHLKGKSIQFLRQVEMEDPVCYIIAGLMLVYPIMLKTLGW 385 b4 330 LHSQQLYRKWLLTCMTSQFSFFARSIQFLRQVEMEDPVCYIIAGLMLVYPIMLKTLGW 387 **b5** 330 LHSQQLYRKWLLTCMTSQVCTR 351 PpSMXLC - Pp3c9_16100 WT 250 ESHERRELVCRAPNRSNPVVGPGGGLSSKDEDVLNILNIFLRPRIKNVILVGDITAANAVN 310 c1 250 ESHERRELVCRAPNS---VVGPGGGLSSKDEDVLNILNIFLRPRIKNVILVGDITAANAVN 310 c2 250 ESHERRELVCRAPNRIRLLDLEVVFHRRMRTSSTF 284 c3 250 ESHERRELVCRALESGCWTWRWSFIEG 276 c9 250 ESHERRELVCRAPN---PVVGPGGGLSSKDEDVLNILNIFLRPRIKNVILVGDITAANAVN 310 c10 250 ESHERRELVCRAPNIRLLDLEVVFHRRMRTSSTF 283 c11 250 ESHERRELVCRAPDSIESGCWTWRWSFIEG 279 WT 1 MRSGANSVQHLLTPAAHGVLKYAVTEARRRGHPQVQPLHVVSMLLTHAGSRLRQACMLSQ 60 Ac5 1 MRSGANSVQHLLTPAAHGVLKYAVTELEFKLV 32 (Alternative in-frame STOP codon) PpSMXLD - Pp3c15_16120 WT 28 RRRGHPQVQPLHVVSMLLTHAESRLRQACMLSHPHNSQAAECRALEVCFNVALDHLPQSALAATSQPILSNAL 100 d1 28 RRRGHPSMLSPCSSLTRPAVCDKRVCYRIRTTLRPQSAGLWRFASMLHWTIFRSPRWLLPASLFCQMP 95 d2 28 RRRGNPSMLSPCSSLTRPAVCDKRVCYRIRTTLRPQSAGLWRFASMLHWTIFRSPRWLLPASLFCQMP 95 d5 28 RRRGHPRCNPSMLSPCSSLTPRAVCDKRVCYRIRTTLRPQSAGLWRFASMLHWTIFRSPRWLLPASLFCQMP 99 d6 28 RRRG--QVQPLHVVSMLLTHAESRLRQACMLSHPHNSQAAECRALEVCFNVALDHLPQSALAATSQPILSNAL 100

Figure VI-6 – Local alignments of predicted PpSMXL mutant protein sequences. Numbers indicate the range of local alignments. WT sequences are given in bold black. Stretches of variant amino acids finishing with a premature STOP codon are written in red. Small insertions/deletions of amino acids are noted in orange. Other Δ mutations are not shown here as mutant alleles cannot generate proteins (see Supplemental Figure VI-10 for genomic DNA alignments).

Ppsmxl loss-of-function mutants do not display a constitutive SL response phenotype

In order to clarify the function of the four *PpSMXL* genes in *P. patens*, we employed two CRISPR-Cas9 mutagenesis strategies. In the first one, we used only one guide RNA targeted against the most upstream possible CDS region. However, since the functional relevance of most of SMXL proteins domains is barely known, we ensured obtaining knock-out mutants by carrying out another CRISPR mutagenesis strategy in parallel with the former. In this second strategy, we used multiple guides, with one in each untranslated region (UTR) (Figure VI-1 and supplementary table). Mutations giving rise to a complete deletion of the CDS are noted as $Ppsmxl\Delta$, while those causing a frameshift are simply noted as *Ppsmxl* (Figure VI-6 and Supplemental Figure VI-10). Reasoning that the four PpSMXL proteins group into two clades, from our previous observations and from literature (Walker et al., 2019), we first sought double mutants for each clade, e.g. $Ppsmxl(\Delta)ab$ and $Ppsmxl(\Delta)cd$ mutants. Our starting hypothesis was that P. patens SMXL proteins could be functionally analogous to the SMXL7/D53 clade of Angiosperms, e.g. they would be acting as negative regulators of SL signaling. Under this hypothesis, *PpSMXL* loss of function should give rise to a growth phenotype opposite to that of the SL biosynthesis mutant Ppccd8, e.g. restricted protonema extension. We noted that all mutants solely disturbed in the AB clade (both double and single mutants) were highly similar to the WT when grown in white light (Figure VI-7, Supplemental Figure VI-11A, Supplemental Figure VI-12A), except in one experiment where all were slightly more extended than WT (Supplemental Figure VI-12B). Therefore, *PpSMXLA/B* have only a minor role in growth in white light and they do not appear to play a negative role in transduction of the PpCCD8-derived signal. On the other hand, both *Ppsmxlcd* and *Ppsmxl\Deltacd* double mutants have markedly enhanced *protonema* extension, resulting in very large plants (Figure VI-7), even surpassing the *Ppccd8* SL biosynthesis mutant in some experiments (Supplemental Figure VI-12A). Simple *Ppsmxl* Δc and *Ppsmxl*d mutants also tended to be larger than WT, although not to the same level as double mutants (Figure VI-7). When grown vertically in the dark, A/B clade mutants tend to grow as many *caulonema* filaments as WT, of similar length (Supplemental Figure VI-13). On the contrary, clade C/D mutants develop more and longer *caulonema* filaments than WT, similarly to *Ppccd8* (Supplemental Figure VI-13). Taken together, these results suggest that (1) *PpSMXLC* and *PpSMXLD* have a repressive effect on *protonema* growth, at least partly by limiting caulonema filaments number and length; (2) PpSMXLC and PpSMXLD act redundantly to limit protonema growth; (3) PpSMXLA and PpSMXLB play a very modest role in regulation of protonema growth; (4) Ppsmxl and $Ppsmxl\Delta$ mutations in the same genes have comparable effects on phenotype. Thus, shortened proteins produced from *Ppsmxl* alleles are not functional, even when most of the D1 domain is (putatively) still present for the PpSMXLA, PpSMXLB and PpSMXLC mutant proteins (Supplemental Figure VI-1).



Figure VI-7 – **Plant extension of** *Ppsmxl* **mutants.** (A) Phenotype of three-week-old plants on low nitrogen content medium (without underlying cellophane). Scale bars are 5mm. (B) and (C) Diameters were measured each week for the same 35-49 plants of each genotype. Statistical significance of differences between mutants and WT at the last time

point are indicated as bold symbols (Kruskal Wallis followed by a Dunn *post-hoc* test, * 0.01≤p<0.05, ** 0.001≤p<0.01, *** p<0.001).

Loss-of-function of all four *PpSMXL* genes causes a dramatic loss of viability

Our first mutagenesis strategy also granted us some higher order *Ppsmxl* mutants, of which we characterized one triple mutant (*Ppsmxla1b1d1*) and one quadruple mutant (*Ppsmxla2b2c1d2*). It should be noted that the *Ppsmxlc1* mutation in the quadruple mutant probably does not cause a loss of function of *PpSMXLC*, as it does not induce a frameshift (Figure VI-6). The triple and quadruple mutants displayed highly restrained growth and browning of the *protonema*, and only rare stunted gametophores were produced (Supplemental Figure VI-11B, Supplemental Figure VI-12A). Moreover, these higher order mutants grew few agravitropic curled *caulonema* filaments in the dark (Supplemental Figure VI-13). These mutants appeared sterile, as the very few capsules formed were empty. Thus, it appears that loss of function of both clades has a dramatic effect on viability, maybe linked to an increase of senescence. Due to the severe growth defect of these mutants, we could hardly work on them anymore after several rounds of vegetative propagation: even when cultures were restarted exclusively from green tissues, growth was increasingly difficult.

Loss of either clade A/B or C/D PpSMXL function leads to (-)-GR24 insensitivity, whereas only the loss of C/D function results in (+)-GR24 insensitivity

We then explored whether $Ppsmxl(\Delta)$ mutants have a modified phenotypic response to (±)-GR24 enantiomers. To investigate this response, we relied on the test of *caulonema* growth in the dark (Hoffmann *et al.* 2014, see also Chapter IV). When treated with 1µM (+)-GR24, WT plants develop significantly less caulonema filaments, while 1µM (-)-GR24 treatment tends to increase filaments' number (Figure VI-8). Consistent with its lack of endogenous SL, the Ppccd8 mutant has more filaments than WT in control conditions, and (+)-GR24 treatment has a stronger repressive effect on *caulonema* number compared to WT. Response to (-)-GR24 is however either completely abolished in *Ppccd8*, or sometimes even tends to be reversed in some experiments (Supplemental Figure VI-14). As for *Ppmax2*-1, it responds as *Ppccd8* with a similar tendency towards a decrease in filaments number after (-)-GR24 treatment (Figure VI-8, Supplemental Figure VI-14). When investigating the response of *Ppsmxl* double mutants to (+)-GR24, it appears that ab mutants can still respond to this molecule, apparently as much as the WT, while the response of clade C/D Ppsmxl double mutants is virtually abolished (Figure VI-8). We can also note that these double mutants develop much more filaments than WT or even *Ppccd8* in the absence of treatment, supporting the negative role of *PpSMXLC/D* in filaments growth. When we look at simple mutants however (Supplemental Figure VI-15), we find that response to (+)-GR24 is not disturbed, suggesting *PpSMXLC* and *PpSMXLD* play redundant roles in this response. Moreover, simple *Ppsmxlc* mutants develop slightly but significantly more filaments than WT in control conditions, which indicates that *PpSMXLC* likely plays a predominant role over *PpSMXLD* in the regulation of *protonema* growth.



Figure VI-8 – Phenotypic response of *Ppsmxl* double mutants to (+)-GR24 and (-)-GR24 in the dark. 24 plants of each genotype were cultivated for two weeks and then treated with 0,01% DMSO (control, grey), 1 μ M of (+)-GR24 (blue) or 1 μ M (-)-GR24 (red). Plants were incubated vertically in the dark for ten days. Negatively gravitropic *caulonema* filaments were enumerated for each plant. Statistical significance of comparisons of control groups relative to WT is shown as bold black symbols (Kruskal Wallis followed by a Dunn *post-hoc* test, * 0.01≤p<0.05, **

 $0.001 \le p < 0.01$, *** p<0.001). Statistical significance of comparisons between control and treated for each genotype is shown as bold blue or red symbols (Mann-Whitney test, * $0.01 \le p < 0.05$, ** $0.001 \le p < 0.01$, *** p<0.001).

As for response to (-)-GR24, we can see that both clade A/B and clade C/D double mutants tend to be less sensitive than WT (Figure VI-8), while none of the simple *Ppsmxl* mutants responds differently to (-)-GR24 (Supplemental Figure VI-14). This observation leads to the hypothesis that all four *PpSMXL* genes play a redundant role in response to (-)-GR24 in this experimental setup (in the dark). Aiming to clarify the involvement of *PpSMXL* genes in the response to endogenous compounds, and investigating this at an earlier time point, we looked at the expression of SL-responsive genes in *Ppsmxl* mutants in the dark. We found that *PpCCD7* expression level, which is highly increased in the *Ppccd8* background (Proust et al., 2011), is not impacted by loss of *PpSMXL* function (Supplemental Figure VI-16). Thus, SL biosynthesis is likely not regulated by PpSMXL proteins. We also compared the levels of expression of *Pp3c6-15020*, a gene that is induced by both (+)-GR24 and endogenous PpCCD8-derived compounds. Contrary to *PpcCD7*, expression of this gene is disturbed in *Ppsmxl* mutants (Supplemental Figure VI-16): It is overexpressed in the simple *Ppsmxld* mutant and (non-significantly) in the simple *Ppsmxlb* mutant as well as in both double mutants. This would imply that *PpSMXL* genes redundantly repress SL response on the short-term, contrary to what is hypothesized in the long-term (*caulonema* growth assays).

PpSMXL loss-of-function does not restore WT growth in the Ppccd8 background

If PpSMXL genes were involved in SL signaling repression, we could also hypothesize that *protonema* extension of the Ppccd8 mutant would be restored to WT levels by the $Ppsmxl(\Delta)$ mutations. However, when we followed the growth of Ppccd8 Ppsmxl mutants (Figure VI-9 and Supplemental Figure VI-17), we noticed that mutation of either the A/B clade or the C/D clade did not restore *protonema* extension to WT levels. More surprisingly, even the mutation of all 4 PpSMXL genes, which had a dramatic negative effect on growth in the WT background (Supplemental Figure VI-11), was completely circumvented by the Ppccd8 mutation (Figure VI-9 and Supplemental Figure VI-17).



Figure VI-9 – **Genetic analysis of** *PpSMXL* **relationship with** *PpCCD8***.** The mutagenesis strategies used on WT were re-enacted in the *Ppccd8* mutant background. (A) Phenotype of three-week-old plants on low nitrogen content medium (without underlying cellophane). Scale bars are 5 mm. (B) Extension phenotype of these mutants and mutants carrying equivalent *Ppsmxl* mutations in the WT background, on low nitrogen content medium (with underlying cellophane), along a 5-week kinetic. Statistical significance of comparisons relative to WT at the last time point is shown as bold black letters (Kruskal Wallis followed by a Dunn *post-hoc* test, p<0.05).



Figure VI-10 – **Growth of** *proZmUbi:GFP-PpSMXL* **lines.** (A) Plant extension in control conditions. Diameters were measured each week for the same 35 plants of each genotype. Scale bars are 1cm. Statistical significance of differences relative to WT at the last time point are indicated as bold symbols (Kruskal Wallis followed by a Dunn *post-hoc* test, * $0.01 \le p \le 0.05$, *** p < 0.001). (B) Growth of gametophores in red light. Gametophore height was measured for 30 two-

month-old gametophores of each genotype. Length of phytomeres was estimated by dividing each gametophore's length by its number of phyllids. Scale bars are 1 mm. Each point represents a measurement. Statistical significance of comparisons between mutants and WT are indicated by bold symbols (Kruskal Wallis followed by a Dunn *post-hoc* test, $*0.01 \le p < 0.05$, *** p < 0.001). OE GFP-S-A=*proZmUbi:GFP-PpSMXLA;* OE GFP-S-C-1=*proZmUbi:GFP-PpSMXLC* line 1; OE GFP-S-C-2 = *proZmUbi:GFP-PpSMXLC* line 2.

PpSMXL overexpressing lines display phenotypes alike Ppmax2-1

To better understand *PpSMXL* function, we examined the phenotype of the *GFP-PpSMXLA* and *GFP*-*PpSMXLC* overexpression lines we previously used to work out the subcellular localization of PpSMXL proteins. We checked the GFP-PpSMXL fusion transcript overexpression (Supplemental Figure VI-18) and for better clarity, we simplified the notation of these lines as OE GFP-S-A and OE GFP-S-C, respectively. When grown under standard conditions in white light, two out of the three lines (OE GFP-S-A and OE GFP-S-C-2) were significantly less radially extended than the WT and displayed a growth phenotype similar to that of *Ppmax2-1* (Figure VI-10 A), with far less but bigger gametophores, that develop earlier than in WT (Supplemental Figure VI-19). Furthermore, when we examined the phenotypic response of these lines to long-term red-light continuous illumination, we likewise found that these OE lines, especially OE GFP-S-A, behaved very similarly to Ppmax2-1 (Figure VI-10 B). When grown in these conditions, the *Ppmax2-1* mutant grows very elongated gametophores, while *Ppccd8* consistently keeps shorter gametophores than WT (Lopez-Obando et al., 2018). Interestingly, we found that the OE GFP-S-C-1 line, which was undistinguishable from WT when grown in control conditions, also developed elongated gametophores in red light (Figure VI-10 B). These features point to *PpSMXLA* and *PpSMXLC* acting downstream of *PpMAX2* and playing an opposite role to *PpMAX2* in development and imply that the PpSMXLA and PpSMXLC proteins could be targets of PpMAX2-dependent proteasomal degradation. We moreover noticed that Ppsmxl mutants, of the C/D clade only, develop significantly smaller, stunted, gametophores compared to WT or even *Ppccd8* in red-light, thus displaying an opposite phenotype to OE-PpSMXL lines in these same conditions (Supplemental Figure VI-20).



Figure VI-11 – **Genetic analysis of** *PpSMXL* **relationship with** *PpMAX2***.** The mutagenesis strategies used on WT were re-enacted in the *Ppmax2-1* mutant background (Lopez-Obando *et al.* 2018). A *Ppmax2 CRISPR* mutagenesis using 5 guide RNAs was employed in parallel in the *Ppsmxl* $\Delta c7\Delta d4$ mutant background, giving rise to the *Ppmax2-16* mutation. (A) Representative individuals for those measured in Figure VI-11-B, at the end of the experiment (week 5).

Scale bars are 1cm. (B) Quantification of the growth of the same mutants on low nitrogen content medium (with underlying cellophane), along a 6-week kinetic. (C) Growth of two *Ppmax2/Ppsmxl* mutants gametophores under constant red light. Gametophore height was measured for 30 two-month-old gametophores of each genotype. Each point represents a measurement. Statistical significance of comparisons between all genotypes are indicated by bold letters (Kruskal Wallis followed by a Dunn *post-hoc* test, p<0.05). Representative gametophores are shown in the picture on the right. Scale bar is 3mm.

PpSMXL mutations partially restore **Ppmax2-1** mutant phenotypes

In order to formally prove this connection between PpSMXL proteins and PpMAX2, we tested whether *Ppsmxl* loss of function could restore a WT phenotype when associated with loss of function of *PpMAX2*. We struggled to obtain *Ppsmxlab* mutants in the *Ppmax2-1* background and we could not obtain any *Ppsmxlcd* mutants at all. Therefore, we switched strategies and instead mutated *PpMAX2* in one of our *Ppsmxlcd* mutants, using the CRISPR-Cas 9 system again, with five guide RNA against *PpMAX2* (Supplemental Figure VI-21). When we examined the *protonema* extension of these multiple mutants, we found that only the association of the *Ppmax2* and *Ppsmxlcd* mutations could partially complement the dramatically decrease in growth caused by *PpMAX2* loss of function (Figure VI-11A and B). On the other hand, in red light, both *Ppsmxlab* and *Ppsmxlcd* mutations could almost completely restore the excessive elongation of gametophores caused by *PpMAX2* loss of function (Figure VI-11C). To be more precise, the *Ppsmxlcd* mutations again have a bigger effect relative to *Ppsmxlab* mutations, as they even decrease phytomere length to levels comparable to *Ppccd8* (Supplemental Figure VI-22).



Figure VI-12 – PpSMXL proteins interact with PpMAX2 and can form homo-oligomers. The first indicated protein is fused to the N-terminal part of eYFP, while the second protein is fused to the C-terminal part (both tags are fused at the N-terminal end of *P. patens* proteins). Both fusion proteins are transiently overexpressed in *N. benthamiana* leaf

epidermis. Colocalization of CFP-H2b and eYFP biFC signals are pointed at with white arrows. Scale bars are 50µm. Interactions with DEFICIENS and GLOBOSA proteins from *Antirrhinum majus* are given as negative controls.

PpSMXL proteins interact with players of the PpMAX2-dependent pathway

In bimolecular fluorescence complementation (BiFC) assays, all four PpSMXL proteins interact with PpMAX2 when respectively fused to complementary parts of the eYFP fluorescent protein and overexpressed in the same *Nicotiana benthamiana* cells (Figure VI-12). Although the eYFP signal is quite low for PpSMXLA and PpSMXLC, we can notice a nuclear interaction of both with PpMAX2 (the previously published GLOBOSA/DEFICIENS interaction was herein consistently used a positive control of eYFP reconstruction, see Bouchez et al., 2008). Interestingly, we also found that PpSMXLB and PpSMXLD could potentially form homo-oligomers. On the other hand, formation of PpSMXL hetero oligomers between themselves remains to be investigated fully, nonetheless we have already found that PpSMXLA was not involved in such structures. In order to confirm these interactions, we tested them *in vitro* by yeast two hybrid (Y2H) assays, where interactions are semi-quantitatively revealed by blue coloration. Unfortunately, PpMAX2/PpSMXL interactions were not observed in yeast, using either protein as the bait or the prey (Supplemental Figure VI-23). Moreover, PpMAX2/PpSMXL interactions were not triggered in this system by addition of (±)-GR24 (even at the high concentration of 50µM). We could not replicate the homo-interactions of PpSMXLB and PpSMXLD either and, while there was a PpSMXLB/PpSMXLD interaction, it was interestingly weakened in the presence of (±)-GR24 (Supplemental Figure VI-23). This interaction is reliable only with PpSMXLD taken as the bait, as slight autoactivation was observed when using PpSMXLB as the bait.

Using the same two techniques, we investigated the interactions of PpSMXL proteins with PpKAI2L proteins. Among the PpKAI2L proteins we tested (at least one from each clade: PpKAI2L-C, -G, -J, -F, -H, -L), only PpKAI2L-C could interact with PpSMXL proteins, moreover the four of them (Figure VI-13). Here again, we had some issue with the replication of these results in Y2H (Supplemental Figure VI-24). Indeed, we had a severe autoactivation problem with most of clade A-E PpKAI2L when taken as baits. Still, we did note that blue coloration was darker when PpSMXLB/C/D (B especially) were used as preys, thus suggesting an interaction between PpSMXLB/C/D and clade A-E PpKAI2L as a whole. This seems consistent with our previous demonstration that PpSMXL proteins act in the PpMAX2-dependent pathway, since clade (A-E) *PpKAI2L* genes are also involved in this pathway (see chapter IV). Additionally, we found PpKAI2L-F appeared to also interact with PpSMXLB/C/D (Supplemental Figure VI-24), which we did not explore in biFC. Another new interaction was PpSMXLB/PpKAI2L-K (Supplemental Figure VI-24), which we did not explore in biFC. Globally, it is interesting to take note that all these interactions are not dependent on (±)-GR24 addition. This implies either that they do not need a PpKAI2L-perceived ligand to occur, or that this ligand (or molecule(s) with the same activity and close structure) is present in yeast. Both biFC and Y2H revealed only weak interactions involving PpSMXLC (despite both methods using overexpression of proteins of interest), while it seems to be the main PpSMXL player in *P. patens*, given mutants phenotypes.



Figure VI-13 – PpSMXL proteins interact with some PpKAI2L proteins. The first indicated protein is fused to the N-terminal part of eYFP, while the second protein is fused to the C-terminal part (both tags are fused at the N-terminal end of *P. patens* proteins). Both fusion proteins are transiently overexpressed in *N. benthamiana* leaf epidermis. Colocalization of CFP-H2b and eYFP biFC signals are pointed at with white arrows. Scale bars are 50µm. Interactions with DEFICIENS and GLOBOSA proteins from *Antirrhinum majus* are given as negative controls.



Figure VI-14 – **Interactome of the PpSMXLC protein highlight its major implication in growth.** The transgenic *P. patens* line (number 1) expressing the *proZmUbi:GFP-PpSMXL* fusion construct was used for a pull down-assay using antibodies against GFP (GFP-trap). 37 proteins were recovered specifically with the GFP-PpSMXLC bait. Annotations of these proteins (by homology with Angiosperms proteins) enabled us to group them by GO (corresponding to the categories shown in the pie chart).

PpSMXLC interactome reveals a major implication in regulation of photosynthesis and protein turnover

In order to clarify the interactions of PpSMXLC, we used a GFP-trap pull-down strategy to recover proteins interacting *in planta* with GFP tagged PpSMXLC, with or without a 6h 3μ M (±)-GR24 treatment, and then identified signature peptides recovered with GFP-PpSMXLC by MS/MS (Figure VI-14). Using this without *a priori* method, we did not recover PpMAX2/PpSMXLC or PpKAI2L/PpSMXLC interactions, suggesting that these interactions are indeed transient and/or too weak to endure the experimental procedure of protein complex recovery here. However, we found 37 signature peptides that were significantly enriched in untreated GFP-PpSMXLC samples relative to untreated flag-GFP samples, which we considered as specific interactors of PpSMXLC. Most of them are involved in photosynthesis, energetic metabolism (glycolysis and mitochondrial respiration) and protein turnover (translation and protein degradation), together making up 72% of interacting proteins (Figure VI-14 and Supplemental Figure VI-25). The remaining proteins are involved in oxidative stress (14%), cytoskeleton (5%), specialized metabolism (4%, notably OPDA and possibly kaurene biosynthesis enzymes) and finally 5% belong to other categories or have no predictable function. (±)-GR24 had virtually no impact on the interactions of PpSMXLC, with only 3 interactions induced by (±)-GR24, and 3 others diminished by (±)-GR24 (Supplemental Figure VI-26).

Discussion

Phylogeny and expression profiles a differential regulation of clade A/B and clade C/D PpSMXL

It has already been shown that most of mosses have an additional SMXL clade compared to other non-seed plants (Walker et al., 2019). We report evidence of this in *P. patens*, in which *PpSMXLA/B* belong to this divergent clade, which could potentially be neofunctional, as we have shown earlier for some PpKAI2-L homologs (see chapter IV). The split between *PpSMXLA* and *PpSMXLB*, and between *PpSMXLC* and *PpSMXLD* on the other hand, is probably quite recent given the high similarity of encoded proteins in each given clade (supplemental Figure VI-1). PpSMXLA/B moreover have shorter D1 and M domains compared to PpSMXLC/D (supplemental Figure VI-1) and lack the typical degron motif (supplemental Figure VI-7). One could argue that the consensus RGKT sequence is also modified in some flowering plants SMXL where the motif is nonetheless functional (OsSMAX1 (Zheng et al., 2020), however among sequences examined, PpSMXLA/B are the only one lacking the K residue, which is functionally relevant in Walker A motifs as it is necessary for phosphate binding (Bianchi et al., 2012). Inversely, the EAR motif is conserved in all four PpSMXL proteins, thus transcriptional regulation could be a uniting feature between the two clades.

Additionally, expression level of *PpSMXLA/B* is lower than that of *PpSMXLC/D* in *protonema*. All four genes are more highly expressed in older gametophores and/or rhizoids (Figure VI-2) and in spores (Supplemental Figure VI-3). This could indicate that the function of the two clades is relevant in stress-resistant organs (aging gametophores and spores), while only the C/D clade has a relevant function in the *protonema*. Moreover, the two clades gene expression displays opposite sensitivity to (±)-GR24 enantiomers: *PpSMXLA/B* transcript levels are slightly induced by (+)-GR24 in a PpMAX2-dependent way, in the dark, while *PpSMXLC/D* are slightly induced by (-)-GR24 in a PpMAX2-dependent way, even more so in the light (Figure VI-3, supplemental Figure VI-4). However, the *C/D* clade becomes induced by (+)-GR24 instead, in the absence of PpMAX2 (*Ppmax2-1* mutant), even more in the light (supplemental Figure VI-4). Puzzlingly, only the *C/D* clade appears sensitive to PpCCD8-derived compounds (SL), in the light, when these compounds are theoretically mimicked by (+)-GR24 (supplemental Figure VI-4). This suggests that natural SL produced by *P. patens* normally repress *PpSMXLC/D* gene expression, which might represent a level of crosstalk between PpCCD8-derived compounds signaling and PpMAX2-dependent signaling. However, all *PpSMXL* genes are regulated in a PpMAX2-dependent manner, especially *PpSMXLA* and *PpSMXLC* which are also the main targets of regulation by light (both on the long-term (Figure VI-2) and on the short-term by red light (Figure VI-3)).

PpSMXL proteins function at least partly involves the nuclear compartment

The presence of predicted NLS sequences, as well as an EAR motif in the four PpSMXL proteins hint at a nuclear localization (Supplemental Figure VI-1, supplemental Figure VI-6). Indeed, all four PpSMXL are located in the nucleus, albeit not only (Figure VI-5, supplemental Figures VI-6,7,8 and 9). Still, this partial nuclear localization is enough to enable the interaction of PpSMXLs with proteins in this organelle (PpMAX2 and PpKAI2L-C, Figures VI-12 and VI-13). It would be interesting in the future to explore whether this EAR motif grants PpSMXL proteins a function in transcriptional regulation. Notably, we could determine if they are able to interact directly with DNA, like has recently been demonstrated for AtSMXL6 (Wang et al., 2020b), and which loci are targeted. Such effect could also

be reliant on PpSMXL interaction with TOPLESS transcriptional repressors (TPL) and we accordingly project to test the interaction between PpSMXL and the two TPL homologs from *P. patens*.

PpSMXL proteins are not highly sensitive to SL levels and their sensitivity to KL probably depends on light

We report herein that none of the four PpSMXL proteins are rapidly degraded in response to (+)-GR24, not even PpSMXLA/B which transcripts are increased by treatment with this enantiomer in the dark (supplemental Figures VI-8 and VI-9). The absence of (+)-GR24 triggered degradation is probably not the result of an insufficient amount of (+)-GR24 and/or treatment duration, as (1) AtSMXL6 is almost completely degraded after 20 minutes of (\pm)-GR24 2µM in p35S:AtSMXL6-GFP seedling (Wang et al., 2015b); (2) AtSMXL7 is significantly decreased after only 10 minutes of a 1µM (\pm)-GR24 treatment in p35S:AtSMXL7-YFP Arabidopsis roots (Soundappan et al., 2015); (3) as little as 12 minutes are necessary for pOsAct:D53-GFP degradation in rice roots (Zhou et al., 2013). Thus, PpSMXL proteins do not behave similarly to AtSMXL6/7 or D53 proteins and they seem insensitive to both the SL mimic (+)-GR24 and endogenous SL from both *P. patens* (GFP lines) and *N. benthamiana* (RFP transient expression lines). While their stability in *N. benthamiana* could be explained by an incompatibility with the Angiosperm MAX2, PpSMXLs are also stable in *P. patens*. This indicates that PpSMXL proteins are likely not degraded in the context of SL signaling. Likewise, deletion of the degron motif (or degron-like for PpSMXLA/B) has no effect since these proteins are stable (supplemental Figure VI-7).

The stability of PpSMXLA and PpSMXLC in *P. patens* lines, in the WT background where PpMAX2 is functional, suggests that these proteins are not highly sensitive to the endogenous compound(s) perceived via PpMAX2 either (putative KL). Still, observation of GFP fluorescence was carried out on *P. patens* lines that were previously incubated in low light conditions or even in the dark for a few hours (Figure VI-5, supplemental Figure VI-8). We also observed that when these lines were kept in high light conditions until observation, the intensity of the GFP signal tended to be lower (and even undetectable for the line overexpressing PpSMXLA). These elements hint at an activation of the PpMAX2-dependent KL signaling pathway by light, which leads to PpSMXL degradation. Hence, reported enhanced expression of *PpKAI2-L* (Lopez-Obando et al., 2016a), *PpMAX2* (Lopez-Obando et al., 2018) and *PpSMXL* genes in the dark (Figure VI-3), would be a mean to keep KL signaling active in the absence of light. A mimic of KL ought to be tested on these lines. Notably, KAR₂ could be used, as we showed that it has similar effects as (-)-GR24 on *caulonema* growth in the dark (chapter IV). The role of the degron motif should be investigated in *P. patens* by generating new GFP-PpSMXLΔRGKT lines, in WT and *Ppmax2-1*, and testing the effect of KL mimics and proteasome inhibitors.

Ppsmxl loss of function mutants contradict PpSMXL acting as repressors of SL response

At first glance, it is evident that clade C/D *Ppsmxl* mutants do not display a constitutive SL response phenotype, instead they are alike the SL deficient *Ppccd8* mutant (Figure VI-7, supplemental Figure VI-12). This could be indicative of either a positive role of PpSMXLC/D in SL signaling or a negative role of these proteins in PpMAX2-dependent signaling. The phenotype of clade A/B mutants is more comparable to WT, which does not rule out the possibility of PpSMXLA/B acting as repressors of SL signaling (Figure VI-7). However, both A/B clade and C/D clade loss-of-

function in the *Ppccd8* background result in mutants that are like *Ppccd8* (Figure VI-9, supplemental Figure VI-17). If PpSMXL proteins were repressors of SL signaling, the loss of repression should circumvent the absence of endogenous SL and we could expect restoration to a WT-like phenotype. Therefore, PpSMXL only play a minor role, if any, in the repression of the SL response. However, the function of the PpSMXLC/D clade does seem necessary for response to (+)-GR24 in dark grown *caulonema* (Figure VI-8), and therefore potentially for response to endogenous SL. This can be linked to the previous observation that endogenous SL apparently repress *PpSMXLC/D* genes expression, with the hypothesis of a negative feedback of SL on their PpSMXLC/D-mediated effects.

PpSMXL are negative actors of the PpMAX2-dependent pathway

Two major points of evidence show that PpSMXL proteins are involved in the PpMAX2-dependent signaling pathway and exert a negative role in this pathway: (1) *P. patens* lines overexpressing *PpSMXLA* or *PpSMXLC* are phenotypically similar to the *Ppmax2-1* mutant (Figure VI-10); (2) Loss of function of either *PpSMXL* clade partially restores developmental disturbances caused by loss of *PpMAX2* function (Figure VI-11). Although lines overexpressing *PpSMXLB* or *PpSMXLD* could not be examined, and we did not obtain triple or quadruple *Ppsmxl* mutants in the *Ppmax2-1* background, we hypothesize that the four PpSMXL proteins act in an additive fashion to repress PpMAX2-dependent signaling.

PpSMXL proteins can indeed interact with components of the PpMAX2-dependent pathway: PpMAX2 itself, but also PpKAI2L-C (Figures VI-12 and VI-13). Moreover, PpSMXL/PpMAX2 interactions could be dependent on PpKAI2-L presence, as these interactions are not observed in Y2H (supplemental Figures VI-23).

Also supporting this conclusion, we found that both clades *Ppsmxl* double mutants cannot respond to (-)-GR24, while a response opposite to that triggered by (+)-GR24 is observed in WT (Figure VI-8). Thus, the PpSMXLC/D clade likely cannot ensure response to (-)-GR24 alone e.g. the two *PpSMXL* clades have a redundant function in the context of PpMAX2-dependent signaling.

PpMAX2 is involved in the induction of *PpSMXL* expression, while PpSMXL proteins are likely targets of PpMAX2-dependent degradation. Therefore, PpSMXL could constitute a level of negative feedback regulation in the PpMAX2-dependent pathway. Since this induction by PpMAX2 is especially prevalent in the dark, negative feedback might be more active in the absence of light. On the other hand, the short-term induction of *PpSMXLA/B/C* by red light also relies on PpMAX2. Hence the PpMAX2-dependent regulation of *PpSMXL* expression needs to be further clarified.

PpSMXL could be a bridge linking SL signaling and PpMAX2-dependent signaling

It is interesting to note that "de-repression" of PpMAX2-dependent signaling (*Ppsmxlcd* mutants) and "silencing" of SL signaling (*Ppccd8* mutant) have very similar effects on developmental pattern of the *protonema* (more extended) and gametophores (smaller) (Figure VI-7, supplemental Figure VI-20). This, together with the opposite effects of (\pm) -GR24 enantiomers on dark grown *caulonema* in WT, indicates that these two pathways likely regulate the same processes in opposite manners. One of these processes, as demonstrated for the *Ppccd8* mutant (Hoffmann et al.,

2014), is probably cell division (this is further discussed in chapter VII). Accelerated cell division rate and filaments branching still must be confirmed in *Ppsmxl* mutants, however it is supported by the observation that double *Ppsmxl* mutants of both clades (tend to) develop more *caulonema* filaments than WT in the dark (Figure VI-8). Furthermore, the observation that phenotypic response to (+)-GR24 cannot occur in the absence of PpSMXLC/D also suggest that SL act at least partially via the PpMAX2-PpSMXL pathway. However, as we have seen herein (and in chapter IV), loss of *PpMAX2* function or loss of function in (almost) all clade (A-E) *PpKAI2-L* does not abolish response to (+)-GR24. Hence, it is evident that SL signaling does not solely rely on the PpMAX2-dependent pathway, and we can hypothesize that perception of SL by PpKAI2-L proteins of the (JGM) clade does not act via a regulation of the PpMAX2-dependent pathway but nonetheless requires functional PpSMXLC/D.

PpSMXLC likely has a specific role among PpSMXL proteins

We noted that higher order *Ppsmxl* mutants display highly disturbed growth of gametophores, loss of fertility and rapid browning of the *protonema*, suggesting that extreme activation of the PpMAX2-dependent pathway is very detrimental to viability. The observation that the *Ppccd8* mutation can circumvent the dramatic effect of *Ppsmxlabd* loss of function (PpSMXLC is probably still functional in the presented mutant) (Figure VI-9), together with our previous hypothesis of SL repressing PpMAX2-dependent signaling, implies that accumulation of PpSMXLC in the absence of other PpSMXL is detrimental to development. Under this hypothesis, in the *Ppccd8* mutant, inhibition of the PpMAX2-pathway by endogenous SL would be lifted and PpSMXLC would be degraded in a PpMAX2-dependent manner.

It is surprising that most of PpSMXLC interactors found by this method are not proteins localized in the nucleus, which supports our previous observation that GFP-PpSMXL localization was not exclusively restricted to the nucleus (Figure VI-5). Recovery of PpSMXLC interacting proteins indeed indicated that PpSMXLC likely represses growth at various levels (photosynthesis, cytoskeleton, protein metabolism) and regulates response to oxidative stress (Figure VI-14). If we hypothesize that PpSMXLC switches cell behavior from physiological growth to oxidative stress response, we can think that PpSMXLC accumulation results in a stress response runaway leading to cell death. Other PpSMXL, most probably PpSMXLD, likely associate with PpSMXLC and resulting oligomers have different targets (this is further discussed in chapter VII). Even though such interactions have not been experimentally shown yet, this would explain why this senescence phenotype is not observed in the *PpSMXLC* overexpressing lines (obtained in the WT background (Figure VI-10), nor in *Ppsmxl* mutants aside from the two mutants where only *PpSMXLC* is still WT (Figure VI-7, supplemental Figure VI-11 and VI-17).

Puzzlingly, we found that the amount of pulled down PpSMXLC was apparently decreased by (\pm) -GR24, suggesting that the PpSMXLC protein might actually be less accumulated in response to this treatment, potentially degraded in response to the (-) component (but this might take longer than the 20 minutes we tested earlier, here 6h). However, this potential degradation could be independent of proteasomal activity, as we did not find ubiquitin to be an interactor of PpSMXLC, unlike what was shown for AtSMXL7 (Struk et al., 2018). Alternatively, the treatment might have not been sufficient to enable recovery of ubiquitin at levels above the identification threshold. This might be due to this experiment having been carried out in light conditions, in which we have noticed many times that response to (\pm) -GR24 enantiomers was decreased (namely *caulonema* growth assays and transcriptional response assays). Despite

the possible technical shortcoming of this experiment, we can note that the interactors found for PpSMXLC clearly support the major implication of this protein in general repression of growth, probably by repressing its interactors' activity. We found that (\pm)-GR24 treatment had virtually no impact on PpSMXLC interactions (supplemental Figure VI-26), which is strikingly different from what was shown for AtSMXL7 (Struk et al., 2018), for which more than 30 interactions were promoted by a 1µM (\pm)-GR24 treatment in the dark. Another notable difference with AtSMXL7 interactors (supplemental Figure VI-25), despite it having a canonical EAR motif and being found in the nucleus. Other interactors of SMXL proteins, such as interactors associated with cytoskeleton, translation, energetic metabolism and photosynthesis. Interestingly, (\pm)-GR24 prevents salt stress damage in rice, by limiting chlorophyll degradation and decrease of the photosynthetic rate, and increasing POD (peroxidase) and SOD (superoxide dismutase) activities (Ling et al., 2020). Thus, we could hypothesize that either D53 or OsSMAX1 (or even both) activity would have the opposite outcome, e.g. favoring decrease of photosynthetic ability and accumulating ROS (reactive oxygen species). These two processes are also highlighted by PpSMXLC interactome, therefore proteins involved in these processes might likewise be ancestral interactors of SMXL proteins.

Methods

Cultivation of *Physcomitrium patens* **Gransden.** Unless otherwise explicitly stated in legends, experiments were always carried out on PpNO₃ medium (corresponds to the minimal medium described by Ashton et al., 1979), in the following control conditions: 25° C during daytime and 23° C at night, 50% humidity, long days conditions with 16 hours of day and 8 hours of night (quantum irradiance of ~80 µmol/m²/s). Multiplication of tissues from young *protonema* fragments prior to every experiment is done in the same conditions but using medium with higher nitrogen content (PpNH₄ medium, PpNO₃ medium supplemented with 2.7 mM NH₄ tartrate). For red light experiments, plants were grown on PpNO₃ medium in Magenta pots at 25°C, in continuous red-light (~45 µmol µmol/m²/s). Cellophanes of appropriate sizes were used for monitoring of *protonema* extension kinetics, as well as for the cultures launched in 6-well plates for gene expression studies (see a detailed protocol in our methods chapter titled "Methods for medium-scale study of the biological effects of strigolactone-like molecules on the moss *Physcomitrella patens*"). Analysis of *caulonema* growth in the dark was performed in 24-well plates, with ~2 weeks of growth in control conditions before incubation (\pm treatment) in the dark and placed vertically for ~10 days (see the same method chapter).

Gene expression analyses by qPCR. Total P. patens RNA were extracted and rid of contaminant genomic DNA using RNeasy Plant Mini Kit and on-column DNAse I treatment (Qiagen), following supplier's indications. cDNA were obtained using the MaximaTM H Minus retrotranscriptase (ThermoFisher), from 50-250 ng of total RNA. cDNA extracts were diluted at 1/5-1/8 before use. RT qPCR was performed in a 384 well thermocycler (QuantStudioTM⁵, ThermoFisher), using SsoAdvanced Universal SYBR Green Supermix (BioRad) and appropriate primers. The thermocycler was programmed to run for 3 min at 95°C, followed by 40-45 cycles of 10 sec at 95°C and 30 sec at 60°C. Each biological replicate was run twice to assess technical variation. Expression of genes of interest was normalized by two reference genes among *PpElig2* (*Pp3c14_21480*), *PpAPT* (*Pp3c8_16590*) and *PpACT3* (*Pp3c10_17080*) (all three are expressed at similar levels (Le Bail et al., 2013)). Relative expression was calculated as $RE = 2-CT_{oene}/2-CT_{ref}$ where CT_{ref} is the mean value of the two reference genes. For the study of *PpSMXL* genes' expression across development (figure 2), WT P. patens was cultivated in petri dishes from fragmented tissues for 6, 11 or 15 days, or in Magenta pots for 35 days. Four biological replicates were used for each timepoint. For the "response to GR24" experiment (figure 3 and supplemental to figure 3), WT, Ppccd8 and Ppmax2-1 were cultivated from fragmented protonema in 6-well plates for 2 weeks in control conditions, then transferred in the dark for one week, and treated with 1 µM (+)-GR24, 1 µM (-)-GR24 or 0.01% DMSO in the dark for 6 hours. Six biological repeats were used for each genotype and treatment. For the "response to light" experiment, 2-week-old WT, *Ppccd8* and *Ppmax2-1* were similarly transferred in the dark for 5 days, and then either kept in the dark for 24 hours, kept in control white light conditions for 24 hours, or placed under constant red-light for 1, 6 or 24 hours. Six biological repeats were used for each genotype and treatment.

Statistical analysis of results. Kruskal-Wallis, Mann-Whitney and *post-hoc* Dunn multiple comparisons tests (details in figures legends) were carried out either in R 3.6.3 or in GraphPad Prism 8.4.2. For some gene expression experiments, data points were excluded based on an outliers' search (Grubb's, α =0.05) on in GraphPad Prism 8.4.2. Unless otherwise defined, used statistical significance scores are as follow: # 0.05≤p<0.1, * 0.01≤p<0.05, ** 0.001≤p<0.01, *** p<0.001. Same letters scores indicate that p≥0.05 (non-significant differences).

Cloning of *PpSMXL* CDS and promoters. Coding sequence of each *PpSMXL* gene was amplified on WT *P. patens* Gransden cDNA, using Phusion DNA polymerase (ThermoFisher), following provided instructions and using primers with attB1 and attB2 extensions (respectively on the forward and reverse primer). A similar strategy was used to amplify promoter sequences (full 5'UTR and 1000 bp upstream, in V3.1 sequences available on Phytozome). Both CDS and promoters were then integrated into the pDONR207 plasmid using BP clonase II mix (ThermoFisher). pDONR207 plasmids containing *PpSMXL* CDS were submitted to PCR-mediated mutagenesis to obtain Δ RGK/RT versions.

CRISPR-Cas9 mediated mutagenesis. Coding sequences of *PpSMXL* and *PpMAX2* were used to search for CRISPR RNA (crRNA) contiguous to a PAM motif recognized by *Streptococcus pyogenes* Cas9 (NGG), using the webtool CRISPOR V4 against *P. patens* genome Phytozome V9 (http://crispor.tefor.net/). crRNAs located in the first third of the coding sequence, with highest possible specificity score, and fewest possible predicted off-targets, were selected. Small constructs containing each crRNA fused to either the proU6 or the proU3 snRNA promoter in 5' U3 or U6 promoter (Collonnier et al., 2017), and to the tracrRNA in 3', encased between attB1/attB2 GateWay recombination sequences, were synthesized by Twist Biosciences. These inserts were then cloned into pDONR207 vectors. Polyethylene glycol–mediated protoplast transformation was performed with multiple pDONR207-sgRNA according to the protocol described by Lopez-Obando et al., 2016. Mutations of the *PpSMXL* genes were confirmed by PCR amplification of *PpSMXL* loci around the recognition sequence of each guide RNA and sequencing of the PCR products. Alternatively, for *PpSMXL* genes, a second strategy was employed where crRNAs were designed in the 5' and 3'UTR sequences, to completely remove the coding sequence of *PpSMXL* genes from the genome when used together. Mutants obtained from this second strategy were genotyped by monitoring the size and sequence of amplicons spanning from the 5'UTR to the 3'UTR.

Generation of *proPpSMXL:GUS*, *proZmUbi:GFP-PpSMXL* and control *proZmUbi:flag-GFP* lines. proPpSMXL:GUS constructs were obtained by LR recombination of pDONR207 plasmids containing PpSMXL promoters with the pMP1301 destination vector previously described (Lopez-Obando et al., 2018). This method could not be employed for the promoter of *PpSMXLA*, which had to be amplified with a NotI forward primer and a AscI reverse primer and was subcloned into the pTOPO-blunt II vector (ThermoFisher), and then directly inserted into a NotI-AscI digested pMP1301. proZmUbi:GFP-PpSMXL constructs were obtained by LR recombination of pDONR207 **PpSMXL** pMP1335 plasmids containing coding sequences with the destination vector (http://labs.biology.ucsd.edu/estelle/Moss_files/pK108N+Ubi-mGFP6-GW.gb). Similarly, the proZmUbi:flag-GFP construct was obtained using the pMP1382 destination vector. These plasmids were used independently to transform WT P. patens Gransden, together with pDONR207 containing sgRNA recognizing Pp108 homology sequences contained in the three pMP vectors and appropriate Cas9 and selection plasmids (Lopez-Obando et al., 2016b). Obtained G418 resistant lines were screened for insertion using PCR (with proPpSMXL forward and GUS reverse, GFP forward and PpSMXL reverse, or proZmUbi forward and GFP reverse primers, respectively).

GUS staining. Two to six independent G418 resistant lines with verified *GUS* insertion into the genome were obtained for each *PpSMXL* genes except *PpSMXLB* and used for histochemical analyses. GUS staining of two-week-old *P. patens* plants was carried out following the protocol detailed by Yuji Hiwatashi on the NIBB PHYSCObase website (http://moss.nibb.ac.jp/protocol.html).

Generation of BiFC constructs. Gateway cloned inserts of genes of interest were integrated into pbiFP vectors using LR Clonase II mix (ThermoFisher). Inserts containing a STOP codon were cloned in pbiFP2 and pbiFP3, those not containing a STOP were cloned in pbiFP1 and pbiFP4 (when possible, all four vectors were obtained for a given gene). Resulting vectors were electroporated into *Escherichia coli* DH10B cells and clones were selected on spectinomycin. In phase integration of the coding sequence relative to the half eYFP tag was checked by sequencing insert's ends.

Agroinfiltration of *Nicotiana benthamiana* **leaves.** pbiFP plasmids containing the genes of interest were electroporated into *Agrobacterium tumefaciens* strain C58C1. Agrobacteria were incubated for 18 hours at 28°C under constant agitation and then pelleted, washed twice, and resuspended in infiltration buffer (13 g/L S-medium (Duchefa Biochemie) and 40 g/L sucrose, pH 5.7 with KOH) to attain an OD600 value of 0.5. To enhance transient expression of RFP-PpSMXL and BiFC fusion proteins, the P19 viral suppressor of gene silencing from tomato bushy stunt virus was co-expressed. Equal volumes of needed bacterial cultures were mixed and infiltrated into the abaxial epidermis of 4–5-week-old *Nicotiana benthamiana* leaves. After incubation at 25 °C (16 h light/8 h dark) for 4 days, leaves were harvested on wet paper and kept in similar temperature and hygrometry conditions for short-term preservation until observation.

Confocal microscopy observations. Fragments of *P. patens proZmUBI:GFP-PpSMXL* plants and infiltrated parts of *Nicotiana benthamiana* leaves were both observed on a TCS SP5 inverted confocal microscopy system (Leica), with a 20X objective. GFP fluorescence was acquired in the 495nm-520nm λ range, eYFP in the 525nm-540nm range, RFP in the 570nm-610nm range and CFP in the 465nm-505nm range. Signals in the 700nm-750nm range were attributed to chlorophyll autofluorescence. Lasers used for excitation have a peak wavelength of 488nm (GFP), 514nm (YFP), 458nm (CFP) and 561nm (RFP).

Yeast two hybrid (Y2H) assays. The DupLEXA yeast two-hybrid system (OriGene Technologies) plasmids pEG202 (LexA-bait fusion), pJG4-5 (B42-HA-prey fusion) and pSH18-34 (high sensitivity LacZ reporter). pEG202 plasmids were transformed into the RFY206 yeast strain, together with pSH18-34. pJG4-5 plasmids were transformed into the EGY48 yeast strain. Transformed strains were mated according to interaction of interest, on yeast extract peptone dextrose non-selective medium (YPD: 2% peptone, 1% yeast extract, 2% agar, 2% D-glucose, pH adjusted to 7.0 with NaOH). Mated cells were then selected by two rounds of growth on YNB glucose (0.43g/L YNB, 5g/L NH4SO4, 0.6 g/L dropout, 1% D-glucose, 30 mg/L L-leucine, 2% agar). For assays, selective YNB galactose X-gal medium was prepared from 2X concentrated YNB galactose medium (2.125g/L commercial YNB, 6.25 g/L NH4SO4, 0.75 g/L dropout (-His-Ura-Leu-Trp)) by mixing 175 mL of 2X YNB galactose, 2X agar solution (4%), 50 mL 20% galactose, 50 mL 10% raffinose, 3 mL L-leucine, 400 μ L X-gal (100 mg/mL) and 50 mL of 10X phosphate buffer (7% Na₂HPO₄;7H20 and 3% NaH₂PO₄). Selected mated cells were pinned on selective YNB galactose X-gal plates, with addition of either 500 μ L of 50 mM (\pm)-*GR24* or 500 μ L of DMSO, and grown for 72 hours at 30°C.

GFP-trap experiment. 200 mL liquid PpNH₄ cultures of WT, *proZmUbi:flag-GFP* and *proZmUbi:GFP-PpSMXLC#1* lines were launched from fragmented 7 day-old *protonema* tissues in 500 mL Erlenmeyer flasks. After 3 weeks of constant gentle agitation in control conditions, 200 μ L of 3 mM (±)-*GR24* or 200 μ L of 0.1% DMSO were added to cultures. For each genotype, three biological replicates were used per treatment. After 6 hours, tissues were harvested by filtering cultures (140 μ m filters) and were immediately flash frozen in liquid nitrogen and grinded with a mortar and pestle. Protein extraction was carried out with solutions described in -and following the- GFP-trap protocol by

Chromotek (RIPA lysis buffer was supplemented with protease inhibitors (cOmplete[™] Mini EDTA-free Protease Inhibitor Cocktail from Roche) and 1mg/mL DNAse I). Cell lysates (volume adjusted to 1 mL with dilution buffer) were kept at -80°C until pull-down. 50 µL of lysate was mixed with 50 µL 2X SDS sample buffer to proceed to SDS page and immunoblot analysis to check for proteins integrity and GFP expression (3H9 rat monoclonal anti-GFP antibody, goat anti-rat antibody). Dilution buffer was removed from cell lysates and the extracts were incubated with GFP-trap A agarose beads covalently coupled with alpaca anti-GFP-nano-antibody (Chromotek), for 2 hours at 4°C under constant gentle agitation (beads were previously washed twice with ice cold dilution buffer). Then, beads were recovered by centrifugation at 500 g for 2 minutes (4°C), washed twice with ice cold dilution buffer, and bound proteins were eluted by mixing the beads with 200 µL of MilliQ water and 50 µL of pure formic acid. Eluted fractions were recovered three times and pooled, then dried in a speedvac. Dry extracts were dissolved in 50 µL of urea buffer (20 mM HEPES pH8.0, 8M urea), DTT was added to the final concentration of 15 mM, and samples were incubated at 55°C for 30 minutes under agitation. Then, IAA was added to the final concentration of 30 mM and the samples were incubated at room temperature for 15 minutes in the dark and under agitation. Samples were diluted two-fold with 20 mM HEPES (pH8.0) and predigested by endo-proteinase LysC (1:100 v/v) for 4 hours at 37° C under agitation. Samples were again diluted two-fold in 20 mM HEPES and digested with trypsin (1:100 v/v) overnight at 37°C under agitation, then acidified with TFA (to 1% final concentration). Peptides were captured using C18 OMIX tips (Agilent), following providers' instructions. Peptide extracts were then characterized by MS/MS analyses.

Graphics generation and statistical analyses. Graphs were generated using GraphPad Prims version 8.4.2. Statistical analyses were carried out either in R (version 3.6.3) or in GraphPad Prism. Tests employed were always non-parametric as normality of distributions and/or homoscedasticity among groups could not be confirmed in most experiments (Kruskal-Wallis tests for multiple comparisons and Mann-Whitney for single comparisons, unless otherwise stated in legends).

Accession Numbers. Sequences used in the present article can be found on Phytozome (*P. patens* Gransden genome, V3.1 version). *PpSMXLA* is *Pp3c2_14220*, *PpSMXLB* is *Pp3c1_23530*, *PpSMXLC* is *Pp3c9_16100* and *PpSMXLD* is *Pp3c15_16120*. *PpMAX2* corresponds to *Pp3c17_1180*, *PpCCD7* to *Pp3c6_21550*, *PpCCD8 to Pp3c6_21520*, *PpAPT* to *Pp3c8_16590*, *PpACT3* to *Pp3c10_17080*, and *PpElig2* corresponds to *Pp3c14_21480*. PpHY5a is encoded by *Pp3c7_11360*, and PpPOR1 by *Pp3c12_20650*. The *KUF* homolog we employed as a SL responsive gene corresponds to *Pp3c2_34130*.

Supplemental data

Supplemental Figure VI-1. Predicted functional domains in PpSMXL proteins.

Supplemental Figure VI-2. Tissular pattern of expression of *PpSMXL* genes.

Supplemental Figure VI-3. Expression of *PpSMXL* genes in *P. patens* tissues according to the eFP-Browser database.

Supplemental Figure VI-4. Expression of *PpSMXL* genes in the *Ppccd8* and *Ppmax2-1* mutants in response to (\pm) -GR24 enantiomers in the light and in the dark.

Supplemental Figure VI-5. Expression of *PpSMXL* genes in response to light in the *Ppccd8* and *Ppmax2-1* mutants.

Supplemental Figure VI-6. In silico predictions of PpSMXL proteins subcellular localization.

Supplemental Figure VI-7. Effect of P-loop deletion on RFP-PpSMXL fusion proteins stability and localization in *Nicotiana benthamiana* leaves.

Supplemental Figure VI-8. Effect of (+)-GR24 on stability and subcellular localization of GFP-PpSMXL fusion proteins in transgenic *P. patens* lines.

Supplemental Figure VI-9. Subcellular localization of RFP-PpSMXL fusion proteins in *Nicotiana benthamiana* leaves in response to a (+)-GR24 treatment.

Supplemental Figure VI-10. Used *Ppsmxl* mutations.

Supplemental Figure VI-11. Growth of other *Ppsmxl* mutants.

Supplemental Figure VI-12. Plant extension of other *Ppsmxl* mutants.

Supplemental Figure VI-13. Growth of *Ppsmxl* mutants in the dark.

Supplemental Figure VI-14. Phenotypic response of *Ppsmxl* simple mutants to (-)-GR24 in the dark.

Supplemental Figure VI-15. Phenotypic response of *Ppsmxl* simple mutants to (+)-GR24 in the dark.

Supplemental Figure VI-16. Expression of SL responsive genes in *Ppsmxl* mutants in the dark.

Supplemental Figure VI-17. Growth of other *Ppccd8 Ppsmxl* mutants.

Supplemental Figure VI-18. Expression of GFP-PpSMXL fusion transcripts in transgenic P. patens lines.

Supplemental Figure VI-19. Growth of *proZmUbi:GFP-PpSMXL* lines.

Supplemental Figure VI-20. Growth of *Ppsmxl* mutants' gametophores in red light.

Supplemental Figure VI-21. Used *Ppmax2* mutations.

Supplemental Figure VI-22. Growth of *Ppmax2 Ppsmxl* mutants.

Supplemental Figure VI-23. PpSMXL proteins interactions with PpMAX and among themselves in Y2H experiments.

Supplemental Figure VI-24. PpSMXL proteins interactions with PpKAI2L proteins in Y2H experiments.

Supplemental Figure VI-25. Detailed list of interactors of the PpSMXLC protein.

Supplemental Figure VI-26. Proteins impacted by (±)-GR24.

Supplementary table VI. Sequences of guide RNAs and primers used in this study.

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Protein	Whole amino acid sequence (Phytozome V3.1)
PpSMXLA	MRSGAAAVONTLSLPAQOVLROAISAARERGHAQVOPLHVAFVLLAHGDPVLROACADTHSOTLHGLHOCHALELCF
	NVALD <mark>RLQQCSSSGSTVNLLGLSNALVAALKRAQAQ</mark> QKRGCPDQQQAPLVMKVELEMVIISILEDPSVSRVMEEAGF
N 9-162	FSQQVKTNIENAMSLSALSQQSNVNHLSQRLSASAGPFGNQPLVILERPLFPTREDSNSRSSEGNAPTQELKLPFCS
D1 165-	${\tt NAGASLPISSLVSTTPGSTEVPKLICCSECTDHFNMEYNQIRVQESAKASTVRKQSSTEDEVVVSSLPSWLQKGKDM}$
348	KTEQERHHSLSQRIQELRRKWQFVCSNSHSERCITMDSSPARDRGLGPPSHNWMKASILNNANNSVLMAANPTPLQS
M 377-	KDMALSSALQPSLPSPLPFWDSGFTSVAMPNGPLNGIIGTSGSELSRVETMEKQVESFHDKEEVLQQGGQISPEYMG
D2 583-	PVPSPRPVSKPITSLPSGGLVPIQNPLSKANASLSSTRPSPLSRLKASFPKPSVAACPLKENILPKPSPLSALKTSN
1019	VSPNPASMVKSPPSLAQKIADKPPLSQLVDISAKIDQHSVFFDHKTADRLKYVHKGLMEKVVWQGKAISTISTFIVN
1010	AQIGKGELKGGAAAAGIWLLLEGPDQVGAKLIAGALAELVVGVAAAPIIFGDLGISKWGKKVEEIDGMQI <mark>KGKI</mark> AVD
	RLASMHGARMSLLLOPPREKETVLOGDMDTSVVRDTTRTSALENPANPMSGGMVLKRKASGELSRALLGVKRKKVST
	PFGRIVALDINI SAEENEAVLAETVTSHEEIATNIGVSDPVLRAVOEVLSPKFCSLVDETVMFEPFDLMGLANWILL
	QLSRACSGLVPKFCTRIEVDFQILEHIVSTTWRTPGGRQAFEGWVEDKLKSCLKAVTANSSVSEDRVLRFAYEGCFS
	KELCLGSELPLQIEVACS
DOGMUTE	
PDHAID	MKSGAAAVQNILILEPAQQVLIKAINNAHGKGHAQVQPLHVAFVLLAHEDPLLKQACADIHSQASHGLQQCKALELGF NVALF <mark>RLDOSSSSSTVHDLGLSNNLVALKRAOTH</mark> OKRGCPDOOODDLLTVKVETEOLIISILEDPSVSRVMKFAGF
N 9-162	OSOKVKTNI ENAIGLSASLOOGSANLPSYKPSAPDNHPLPLLMNSANGLYGNOOFATLEKPSFVLKEDINLRSLKVN
D1 165-	AHDOESRLSLPLVGCMGTTASMSPLEWDTLEATADKSYVCCSECMAHYNAECKOIOAODSGOAPICOSPSSEGEGTV
358	TPLPSWLRRAKDMKSELEMPQLHSQQLYRKWLLTCMTSHPERQVNSVSSPGRDGGPSLLHHSWINASIPNNAENSGL
M 387-	$\underline{VIPKS} TPLQSKDMTPPPTQKPNLSSPLPHWARGLSSAAVPDCSLTSNAGTADSKLFRVETAEKQVESYQANGKISRQ$
568	${\tt EGQGLPEDVGPVSSPSPLSKLNASLSITSPSPLSKLSASLLIGKSNPAPSPSPLSKIGSSIPSTNPSPLSKLKASLP}$
D2 609-	KSSASSCPLKGNVLSRPSPLSVLKTSKPSSNSPLKGNFTLVRPQNLSPKPSLNQPVDILAKDDQPSLFDHKAAERLK
1040	HVYKGLLDRVAWQGRAVSTIATSIVNAQTGRGALRGNISRADTWLLLLGPDQVGKRLVVDALAELVLGVGAKPICFG
	DLGFSRWGRKVEETDGLQL <mark>RGRT</mark> AEDSVADAIRANPLSILFLEDIDQADFVLRSKLTKAMETGKFSDSNDRHISVGN
	SITIMTSKLGADCNSGIGKENTFSEARVSSLNGARMSLLLRPPREQEVVFQGDSDISVVRDTISASLLEKPANLLFE
	VDCAVVFERFDLMGOANWLLSOLSSACFGLTPKVCSRTEVDFOLLEHMVSTTWRTPNGRLAFENWVFKKLKSSLNAF
	TSHSGVIENRVLRFAYEDSFSKELCSSSELPLQIEIGCS
DOGMATC	
I PSHAIC	MRSGANSVOILLIPAARGVLKIAVIEARRRGHPUVOPLRVVSMLLIHAGSRLROACMLSOPONSOSAECRALEVCIN VALDHIPOSALAAASOPTISNALMAALKEAHAHORRGCDEHOOSPLLAVKVETDOLTISTLDDPSVSRVMKEAGLSS
N 9-165	TNVKFNLEDGPVTATVANGDGKGLTSHVSNGVGOALSSHEOASKHGIDMVSOGPFYDNSCVTOEENETKASVKFSRL
D1 180-	PANQTSSMESWQQSDAVIESHERRELVCRAPNRSNPVVGPGGGLSSKDEDVLNILNIFLRPRIKNVILVGDITAANA
469	<u>VN</u> SDLALRIKNGNVPAQLQGLQILDPLLSSSSFGYCSSLEMDQKLAELSKIVGECMPAGAILHIGDLQWLAEPMQLK
M 541-	${\tt KGPSNFCPAQRTASELRQLLIRHASSRLWFVGVATPQTFSRLQVLYPSLIADWGLQPVPLSIGSQPDFLSRLTNCTR}$
774	${\bf VVHDLHS} {\tt LHSTPSASVESPRVTLMQDTRPVSNSGPNERFQCCVECLAKFEEERRLIHEHESLSLHLSLTGDEASFGV}$
D2 819-	VNGGTGQVGKESVVQQLMQLREKWQKNCRMLHGDSSQSVGAQSRPQLPSPFSRMTASSHINLPKSCGLGVDVSSRWS
1265	GPGNRSVTSPAVVQPIRASPLFTMNAARQGAISPLQRMVQAQDAASLGADKILEANGGPDTGSPKDGSSVASAPDDH
	EDPHTSTGTSAQNASLANIHTNLALGRANDASPMAGKLRAPISLASKPSPLHAGGAGVGACPISVKNTTKPHWLLQS
	TTVMKCRSGMGSFRGATAKTDTWLLLLGPDPVAKVATAKALAEMVFGGERSLLHTGFADGSPARLEGDDSGMRYRGK
	TPLDRLAEAVRLKPSSVILLEDIDKATSVFKNNVVRAMERGKLADSSMREVSLSNSIIVMTTSVGSVDCEPVERLGA
	LSFSEAKLAALKRAEICVRIKHSSSEKIVFKSANNKIVVVDHGEEEQKKFVETTSSPLEVPL <mark>WVTKRKQDSFLQGEL</mark>
	RSKFDAKRTKSGQGRFLN <mark>LDLNL</mark> STGENKGCWTGETDDHCVTDFDAEEVKRKKVLEHVRLMLTDKFCALPDYAVGFD
	PYDFNGLATEILNTLSKSFEDHSPSEAGVEVDLRLLEYLMSCVWKIPDGRQKFNAWVDDVFSKSISRSLVDISTAGG
	PVVELIAGLSVVKDAFEGVALPHSINFVSTVAV
PpSMXLD	MRSGANSVQHLLTPAALDVLKHAVTEA <u>RRRGHPQVQ</u> PLHVVSMLLTHAESRLRQACMLSHPHNSQAAECRALEVCFN
	VALDHIPQSALAATSOPILSNALMAALKRAHAHORRGCPEHQQSPLLAVKVEIDQLIISILDDPSVSRVMKEAGFSS
N 9-165	${\tt TNVKFNLEDGP} {\tt STVTVANGVEHLINGHVTIGSGQLPNGSEQASKHGNDMVSKGSFYDNSCMTHEENETKTSAKLSSG}$
D1 180-	VMNQANSMGSWQQSDASIGSHERRELAIKTPNSSNRLVGPGASLPLRDEDVLNILEIFLKPKNRNVILVGDTMAANN
469	VSSNLALKIKHGNVPAQLQCLQFLDPQLSSSSFGYCSSLEIEQKLAELSKIVEECMPAGAILHIGDLQWLAEPMQLK
M 535-	KGPSNFCPAQKTASELCQLLIKHASNRLWFIGVATPQIFARLQVLHPSLLADWGLQPVQISAVSQPNLLSRFANNTQ
D2 817-	OT KKOZA V U S POLOGO TU DAVI V SIMOAT DA DĂC CI DE VAU PERENTI LE LE SPORTA A COLUTULA CEMACI EMP
1260	OLASCAVDOPFRTSPSFIMNNSROSIVGSLOGVVEVODAAALGADKSSDAPDEPDLGSPNDGSSVDSAPDDPEGPOG
	STGTSAQIAFLAPIHTNLALGRANDAFLMAGKLRAPISLAMKPSPLHAAGLGPGAGTGASTISLKNNSKPHWLLOSP
	LHQRRAALNSTPRPGGLCGLSSASPLARRLTRLSKSPSPPPVALKEVVDDPTLKGLYKGLMQRVPWQSGAVAGIAAT
	IMKCRSGMGNFRGATAKTDTWLLLLGPDSVAKVAIAKALAEMVFGGERSLIHLGFADGSPARLETDDFRM <mark>RGKT</mark> PLD
	RLVEAVRMKPSSVLLLEDIDKATSVFRNSIVRAMERGKLADSSMREVSVSNTIVVMTSSVGSEEYELEKNSENFTFS
	EAKLAAMGRIDVRARIKNESSGKVVFKGHNDKFFVVDHSELHPNESIGTSFSQMEVPTWVSKRKSDSWFPGQF <mark>RPKV</mark>
	EVKRTKPPEGRFINIDINLAAGESKCNCAVKTDYHCANEFDDEVKREKVLEQARIMLSEKFCALPDYAVGLDSYDFN
	GLGMEILYKLCKSLEDHAPPEVGVEIDLRLLEYIISCVWRIPGGRNKFNAWIDDVFAMSISRALADISNYEGSVVEF
	VADLPEVKDAFEGVALPHSINFASTMAV

Supplemental Figure VI-1– Predicted functional domains in PpSMXL proteins. Domains boundaries were predicted from whole sequence alignments with D53 and searches in the Pfam domain repository. N = Double ClpN domain, D1 = first ATPase domain, M = middle domain, D2 = second ATPase domain. Underlined sequences are those presented in Figure VI-6 alignments. Predicted NLS (see also Supplemental Figure VI-6) are highlighted in cyan. The core degron motif (RGKT) and the EAR motif are highlighted in yellow.



Supplemental Figure VI-2 – **Tissular pattern of expression of** *PpSMXL* **genes.** GUS staining of 2-week old *proPpSMXL:GUS* transgenic plants. Scale bars are 500 μ m unless otherwise stated. (a) and (i) Entire plants. (b), (q) and (v) gametophores. (f), (j) and (r) buds. (h), (t) and (x) close-ups on phyllids. (c), (d), (e), (g), (k), (l), (m), (o), (p), (s), (u) and (w) close-ups on *protonema* filaments or rhizoids. (n) close-up on the base of a gametophore and basal rhizoids.



Supplemental Figure VI-3 – Expression of *PpSMXL* genes in *P. patens* tissues according to the eFP Browser database. Tissular expression pattern for *PpSMXLA*, *PpSMXLB*, *PpSMXLC* and *PpSMXLD*. Results taken from the http://bar.utoronto.ca/efp_physcomitrella/cgi-bin/efpWeb.cgi website, in absolute mode.



Supplemental Figure VI-4 – Expression of *PpSMXL* genes in the *Ppccd8* and *Ppmax2-1* mutants in response to (±)-GR24 enantiomers in the light and in the dark. Transcript levels of the four *PpSMXL* genes, relative to the two reference genes *PpElig2* (*Pp3c14_21480*) and *PpAPT* (*Pp3c8_16590*). Two-week-old plants were incubated in the dark
for one week and then treated with 1μ M (+)-GR24 (diagonal hatches) or (-)-GR24 (horizontal hatches) or DMSO (control, solid color) for 6 hours, also in the dark (grey background). The same experiment was repeated in white light (white background). For each treatment, six biological replicates and two technical replicates were used. Points are the mean of the two technical replicates, coloured bars represent medians. 2-fold differences in median values are estimated as significant (DE). Stars indicate the statistical significance of control levels comparisons between WT and mutants (Kruskal Wallis followed by a Dunn *post-hoc* test, red: mutant>WT, blue: WT>mutant).



Supplemental Figure VI-5 – Expression of *PpSMXL* genes in response to light in the *Ppccd8* and *Ppmax2-1* mutants. Transcript levels of the four *PpSMXL* genes, relative to the two reference genes *PpElig2* (*Pp3c14_21480*) and *PpAPT* (*Pp3c8_16590*). Two-week-old plants were transferred in the dark for 5 days and then either left in the dark for 24h more, put in red light (RL) or in white light (WL) for 1, 6 or 24 hours. For each treatment, four (*Ppmax2-1*) or six (*Ppccd8*) biological replicates and two technical replicates were used. Points are the mean of the two technical replicates, coloured bars represent medians. 2-fold differences in median values are estimated as significant (DE). Some points were excluded from analysis following an outliers identification test carried out in GraphPad Prism (version 8.4.2). Statistical significance of the comparison of mutants' levels in the dark to WT is indicated by stars (Kruskal-Wallis test followed by a Dunn *post-hoc* test, * $0.01 \le p < 0.05$, ** $0.001 \le p < 0.01$, *** p < 0.001, red: mutant>WT, blue: WT>mutant).

Α	AtSMXL7 (85-117): RLPSSKSTPTTTVEEDPPVSNSLMAAIKRSQATAtSMXL6 (85-114): RLPSSKS-PATEEDPPVSNSLMAAIKRSQAN28/30PpSMXLA (83-113): RLQQCSSSGS-TVNLLGLSNALVAALKRAQAQ14/31PpSMXLB (83-112): RLPQSSSSS-TVHPLGLSNALVAALKRAQTH15/30PpSMXLC (82-110): HLPQSALAAASQPILSNALMAALKRAHAH11/29PpSMXLD (82-110): HLPQSALAATSQPILSNALMAALKRAHAH13/29OsSMAX1 (99-131): RLPAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA						
в	Protein	Prediction 1	Prediction 2				
	AtSMXL7	Nucleus (14.3)	Cytoplasm (14.09)				
	PpSMXLA	Nucleus (11.33)	Mitochondria (10.69)				
	PpSMXLB	Chloroplast (12.52)	Nucleus (12.31)				
	PpSMXLC	Nucleus (12.2)	Chloroplast (11.64)				
	PpSMXLD	Chloroplast (11.99)	Cytoplasm (10.87)				
	OsSMAX1	Nucleus (19.18)	Chloroplast (13.06)				
С	Protein	Bipartite NLS (Best)					
	AtSMXL7	828 GPNKRRQEEAETEVTELRALKS	QR (7.9)				
	PpSMXLA	829 VLKRKASGFLSRALLGVKRKKVS (9.9)					
	PpSMXLB	852 EGQLLKRKPSGFLSRALKKCSTP (5.5)					
	PpSMXLC	1069 WVTKRKQDSFLQGELRSKFDAK	RTKSGQG (5)				
	PpSMXLD	1080 RPKVEVKRTKPPEGRFLNLDLNL	AAGESKCNCA (5.2)				
	OsSMAX1	845 DKQVKHRADWLCDDVRPAKLAK	(E (13.5)				

Supplemental Figure VI-6 – *In silico* predictions of PpSMXL proteins subcellular localization. (A) Alignment of the region characterized as a functional NLS in both rice SMAX1 (Choi *et al.* 2020) and Arabidopsis SMXL7 (Liang *et al.* 2016). Numbers in parentheses indicate the range of aligned sequences (in amino acids). Numbers in bold give the ratio of conserved amino acids in the given alignment relative to AtSMXL7. Residues that are conserved between all seven SMXL are in red, those that are conserved in at least two SMXL (relative to AtSMXL7) are in blue. (B) Predictions of SMXL subcellular localization according to ngLOC (http://genome.unmc.edu/ngLOC/).(confidence scores are given in brackets). (C) Predicted NLS according to NLS mapper. Bipartite NLS with a long linker were included in the search, across the whole proteins (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi) (confidence scores are given in brackets and localization of the predicted signals relative to the N-terminal methionine, is given in bold).

А					
CFP	RFP	Merge	CFP	RFP	Merge
a PpSMXLA		Con an	e PpSMXLC		
b PpSMXLA ΔRGRT		2222	f PpSMXLC ΔRGKT		
с РрSMXLB	- 10		g PpSMXLD		
d PpSMXLB ΔRGRT	A STATE		h PpSMXLD ARGKT		1275 2275 2775 2775 2775 2775 2775 2775
В	Walker A consensus	XGXXXXGXGK	TXXXX		
	AtSMAX1	GDGNSSFRGK	TALDK 6/1	15	
	OsSMAX1	DGPNMGFWGK	TALDR 5/1	15	
	PsSMAX1	DSDAHHIRGK.	TVLDR 8/1	15	
	AtSMXL6	CSLDDKFRGK	TVVDY 13	/15	
	AtSMXL7	DSLDDRFRGK	TVVDY 15	/15	
	OsD53/OsD53L	DWDDSSFRGK	GIDC 8/1	15	
	PsSMXL6	VYECHNMLGK	TVVDY 7/1	15	
	PsSMXL7	SFSYDVIIRK	T VVDY 7 /	15	
	PpSMXLA	EIDGMQYRGR	ravds 5/1	15	
	PpSMXLB	EIDGLQLRGR	raeds 4/1	15	
	PpSMXLC	DDSGMRYRGK'	PLDR 7/1	15	
	PpSMXLD	ETDDFRMRGK	PLDR 5/1	15	

Supplemental Figure VI-7 – Effect of P-loop deletion on RFP-PpSMXL fusion proteins stability and localization in *Nicotiana benthamiana* leaves. (A) Infiltrations were carried out in a *N. benthamiana* line stably expressing H2b-CFP (false color here is blue, first picture of each tryptic). Tryptics are p35:RFP-PpSMXLA (a); p35:RFP-PpSMXLA- $\Delta RGRT$ (b); p355:RFP-PpSMXLB (c); $p355:RFP-PpSMXLB-\Delta RGRT$ (d), p355:RFP-PpSMXLC (e); p355:RFP- $PpSMXLC-\Delta RGKT$ (f); p35S:RFP-PpSMXLD (g); and $p35S:RFP-PpSMXLD-\Delta RGKT$ (h). (B) Local alignment of the Walker A/P-loop motif of the second ATPase domain of SMXL proteins from Arabidopsis (At), rice (Os), pea (Ps) and *P. patens* (Pp). Numbers in bold give the ratio of conserved amino acids in the given alignment relative to AtSMXL7. Residues that are conserved between all seven SMXL are in red, those that are conserved in at least two SMXL (relative to AtSMXL7) are in blue.



Supplemental Figure VI-8 – Effect of (+)-GR24 addition on stability and subcellular localization of GFP-PpSMXL fusion proteins in *protonema* of transgenic *P. patens* lines. Control treatment is DMSO 0.01%. Treated filaments were incubated in a solution of (+)-GR24 1 μ M (diluted in 0.01% DMSO) for 20 minutes. Scale bar is 50 μ m.



Supplemental Figure VI-9 – Subcellular localization of RFP-PpSMXL fusion proteins in *Nicotiana benthamiana* leaves in response to a (+)-GR24 treatment. Infiltrations were carried out in a *N. benthamiana* line stably expressing H2b-CFP. Leaf pieces were immerged in a 5μ M (+)-GR24 solution (diluted in 0,1% DMSO) for 20 minutes before observation.

А		WT 934	CAGGAGAGGCACCATTC	(GCTTTCACAGAGAATACAAG	972
		al	CAGGAGAGGCACCATTC			
		a2	CAGGAGAGGCACCATT		TTCACAGAGAATACAAG	
		a3	CAGGAGAGGCACCATT		TCACAGAGAATACAAG	
		a4	CAGGAGAGGCACCATTCTCTG	TGAAATTTCCTTGTA	CTTTCACAGAGAATACAAG	
		a10	CAGGAGAGGCACCATTC	TC	GCTTTCACAGAGAATACAAG	
PpSMXL	A	WT 125	TTGTGCTGCTGGCTCATGGAG	ATCCCGTGC-TACGC	CAGGCATGTGCTGATACGC	A 179
Pp3c2_14	220	a5	TTGTGCTGCTGGCTCATGGAG	ATCCCGTGCCTACGC	CAGGCATGTGCTGATACGC	Ŧ
		a6	TTGTGCTGCTGGCTCATGGAG	ATCCCGTGC	TGATACGCA	f
		a7	TTGTGCTGCTGGCTCATGGAG	ATCCCGTG	AGCCATGTGCTGATACGC	f
		a8	TTGTGCTGCTGGCTCATGGAG	ATCCCGTGC	-CAGGCATGTGCTGATACGC	Ŧ
		a9	TTGTGCTGCTGGCTCATGGAG	ATCC	-AGGCATGTGCTGATACGC	f
		a11	TTGTGCTGCTGGCTCATGGAG	AGC	CAGGCATGTGCTGATACGC	f
		a12	TTGTGCT	GC	CAGGCATGTGCTGATACGC	Ŧ
		WT 1028	B GTATGACCTCACACC		CTGAAAGGCAAGTCAA	1060
		b1	GTATGACCTCACACCTTTCG	ATTCAATTTGTGACA	ATTCA-AAAGGCAAGTCAA	2
DECMIN		b2	GTATGACCTC	GI	GAAAGGCAAGTCAAGTCAA	1
PPSMXL	_D	ЬЗ	GTATGACCTCACACC		TGAAAGGCAAGTCAAT	1
Pp3c1_23	3530	b4	GTATGAC	AAGTCAATTC	AGTTTCTTCGCCAGGTCAAT	?
		FIE 100				1000
		WT 1028	GTATGACCTCACACCCTGAA		GTTTCTTCGCCAGGTAGAGA	A 1082
	•	00	GIAIGACCICACA		AGIAIGIACCAGGIAGAGA	7
		WT 779	GAGCTCCGAAT-AGATCGAAT	CCGGTTGTTGGACCI	GGAG 817	
		c1	GAGCTCCGAAT	CGGTTGTTGGACCI	GGAG	
		c2	GAGCTCCGAATCGAAT	CCGGTTGTTGGACCI	GGAG	
DECAN	~	c3	GAGCTCTCGAAT	CCGGTTGTTGGACCI	GGAG	
PPSMAL	100	c9	GAGCTCCGAAT	GGAG		
Pp3c9_16	5100	c10	GAGCTCCGAATAT	CCGGTTGTTGGACCI	GGAG	
		c11	GAGCTCCGGATTCGATCGAAT	CCGGTTGTTGGACCI	GGAG	
		WT 53	TGTCTTGAAGTATGCGGTTACC	GAGGCTCGGAGGAG	GGTCACCCTCAGGTGCAAC	C 110
		∆ c5	TGTCTTGAAGTATGCGGTTACC	GAGdeleted	d-until-3'UTR	-
		WT 75	CGAGGCACGGAGAAGGGGCCAC	CCCCAGGTGCAACCO	CCTCCATGTTGTCTC 125	
DnSMVI		d1	CGAGGCACGGAGAAGGGGCCA-		CCTCCATGTTGTCTC d1	
Pp3015 16	XLD di	d2	CGAGGCACGGAGAAGGGGC	AACC0	CCTCCATGTTGTCTC d2	
1 p3c13_10	120	d5	CGAGGCACGGAGAAGGGGCAAC	CCC-AGGTGCAACC	CCTCCATGTTGTCTC d5	
		d6	CGAGGCACGGAGAAGGGGCCA-	GGTGCAACCO	CCTCCATGTTGTCTC d6	
в						
-						
DESMALE	WT -15	54 <u>AAAT</u>	TGCCTTGCTAAGTCTCCGGAGA	GGG/4329nt/CTT	CCTACCGCTGCTTAGTTTAG	GAC +122
Ррымхсв	∆.06 ∧1-7	AAAT	TGCCTTGCTAA	//		GAC
I		AAAT	16CC116C	///	TTAGTTTAG	GAC
	WT -20	07 GTTC	ACGCTCTAAAACGAGGTGGTTG	TTA/5039nt/CAG	CCTTCTCTGTAGACTAGCTC	TGC +442
	∆ c4	GTTC	AC	//	GCTC	TGC
PpSMXLC	∆ c6	GTTC	ACGCTCTAAAA	//	CTAGCTC	TGC
,	∆c7	GTTC	ACGCTCT	//	CTGTAGACTAGCTC	TGC
	∆ c8	GTTC	ACGCTCTAAAACGTAGG	//	CTGTAGACTAGCTC	TGC
•						
	WT -41	12 GGAG	CGACACTGGTTTCTGTGGG/55	16nt/AGGTGCTCT	ATCCGATCACGGGGTAGCCT	TCT +408
PpSMXI D	∆ d3	GGAG	CGACACTGGT/	/	T	TCT
	∆ d4	GGAG	CGACAC/	/	GGGGTAGCCT	TCT
	∆ d6	GGAG	CGAC/	/	GGGGTAGCCT	TCT

Supplemental Figure VI-10 – Used *Ppsmxl* and $\Delta Ppsmxl$ mutations. WT sequences are given in bold and guide RNA are underlined. (A) *Ppsmxl* sequences, numbers refer to the position in the CDS relative to the start codon. (B) $\Delta Ppsmxl$ sequences, numbers refer to the position in the UTRs relative to the start and stop codons (- for the 5'UTR and + for the 3'UTR).



Supplemental Figure VI-11– Growth of other *Ppsmxl* **mutants.** (A) Phenotype of three-week-old plants on low nitrogen content medium (without underlying cellophane). Scale bars are 1mm. (B) Phenotype of two-week-old plants on low nitrogen content medium (with underlying cellophane). Scale bars are 2mm.



Supplemental Figure VI-12 – Plant extension of other *Ppsmxl* mutants. (A) and (B) Diameters were measured each week for the same 36 plants of each genotype. Points indicate these individual measurements. Statistical significance of comparisons relative to WT at the last time point are indicated as bold symbols (Kruskal Wallis followed by a Dunn post-hoc test, $* 0.01 \le p \le 0.05$, $** 0.001 \le p \le 0.01$, $*** p \le 0.001$).



Supplemental Figure VI-13 – **Growth of** *Ppsmxl* **mutants in the dark.** Plants were grown in control conditions for 10 days and then placed vertically in the dark for 10 more days. (A) On pictures are representative individuals at the end of the experiment. Scale bars are 2 mm. (B) *Caulonema* filaments number and length was measured for each plant (length corresponds to the mean of the 3 longest filaments of each plant). n=41-48 plants. Points indicate individual measurements. Statistical significance of differences between mutants and WT are given by bold symbols (Kruskal Wallis followed by a Dunn post-hoc test, *** p<0.001).



Supplemental Figure VI-14 – Phenotypic response of *Ppsmxl* simple mutants to (-)-GR24 in the dark. 24 plants of each genotype were cultivated for two weeks and then treated with 0,01% DMSO (control, grey) or 1 μ M (-)-GR24 (red). Plants were incubated vertically in the dark for ten days. Negatively gravitropic *caulonema* filaments were enumerated for each plant. Statistical significance of comparisons of control groups relative to WT is shown as bold black symbols (Kruskal Wallis followed by a Dunn *post-hoc* test, * 0.01 \leq p<0.05, ** 0.001 \leq p<0.01, *** p<0.001). Statistical significance of comparisons between control and treated for each genotype is shown as bold red symbols (Mann-Whitney test, * 0.01 \leq p<0.05, ** 0.001 \leq p<0.01, *** p<0.001).



Supplemental Figure VI-15 – Phenotypic response of *Ppsmxl* simple mutants to (+)-GR24 in the dark. 24 plants of each genotype were cultivated for two weeks and then treated with 0,01% DMSO (control, grey) or 1 μ M of (+)-GR24 (blue). Plants were incubated vertically in the dark for ten days. Negatively gravitropic *caulonema* filaments were enumerated for each plant. Statistical significance of comparisons of control groups relative to WT is shown as bold black symbols (Kruskal Wallis followed by a Dunn *post-hoc* test, * 0.01 \leq p<0.05, ** 0.001 \leq p<0.01, *** p<0.001). Statistical significance of comparisons between control and treated for each genotype is shown as bold blue symbols (Mann-Whitney test, * 0.01 \leq p<0.05, ** 0.001 \leq p<0.01, *** p<0.001).



Supplemental Figure VI-16 – Expression of SL responsive genes in *Ppsmxl* mutants in the dark. Transcript levels of SL responsive genes, relative to the two reference genes *PpElig2* (*Pp3c14_21480*) and *PpAPT* (*Pp3c8_16590*). Expression of *PpCCD7* (*Pp3c6_21550*) is repressed by SL, whereas that of *Pp3c6_15020* is induced by SL. Two-week old plants were transferred in the dark for one week. For each genotype, six biological replicates and two technical replicates were used. Points are the mean of the two technical replicates, grey bars represent medians. Statistical significance of comparisons between mutants and WT is shown as bold black symbols (Kruskal Wallis followed by a Dunn *post-hoc* test, $\# 0.05 \le p < 0.1$, $* 0.01 \le p < 0.05$, $** 0.001 \le p < 0.01$, *** p < 0.001). Some points were excluded from analysis following an outliers identification test carried out in GraphPad Prism (version 8.4.2).



Supplemental Figure VI-17 – Phenotype of *Ppccd8 Ppsmxl* **mutants.** (A) Phenotype of three-week-old other *Ppccd8 Ppsmxl* mutant plants on low nitrogen content medium (without underlying cellophane). (B) Extension of three-week-old mutants alone without underlying cellophane or grouped by five with an underlying cellophane (A) and (B) Scale bars are 5mm.



Supplemental 2 to figure VI-18 – Expression of *GFP-PpSMXL* fusion transcripts in transgenic *P. patens* lines. Semi-quantitative PCR on transgenic lines cDNA extracts.



Supplemental Figure VI-19 – **Growth of** *proZmUbi:GFP-PpSMXL* **lines.** Gametophore number was assessed on sixweek-old plants from the experiment presented in figure 10, panel A (n=14-21 plants for each genotype). Statistical significance of differences relative to WT are indicated as bold symbols (Kruskal Wallis followed by a Dunn post-hoc test, *** p<0.001). OE GFP-S-A= *proZmUbi:GFP-PpSMXLA;* OE GFP-S-C-1= *proZmUbi:GFP-PpSMXLC* line 1; OE GFP-S-C-2 = *proZmUbi:GFP-PpSMXLC* line 2.



Supplemental Figure VI-20 – Growth of *Ppsmxl* mutants' gametophores in red light Gametophore height was measured for 30 two-month-old gametophores of each genotype. Length of phytomeres was estimated by dividing each gametophore's length by its number of phyllids. Each point represents a measurement. Statistical significance of comparisons between mutants and WT are indicated by bold symbols (Kruskal Wallis followed by a Dunn *post-hoc* test, $* 0.01 \le p < 0.05$, *** p < 0.001). Scale bar is 3mm.



Supplemental Figure VI-21 – **Used** *Ppmax2* **mutations.** (A) Genomic sequences. The WT sequence is given in bold green and numbers refer to the position in the *Ppmax2* gene relative to the start codon (in purple). Guide RNA are underlined. (B) Predicted protein sequences. The end of the putative Fbox domain is written in bold blue. The *Ppmax2-1* mutation has been obtained and described previously by Lopez-Obando *et al.* (2018). (C) Location of sequences recognized by thefive guide RNAs used for CRISPR-Cas9 mediated mutagenesis of *PpMAX2* (guide RNAs are named according to their location relative to the ATG and according to their orientation).



Supplemental Figure VI-22 – Growth of *Ppmax2 Ppsmxl* **mutants.** (A) Phenotype of three-week-old plants grown on low nitrogen content medium (without underlying cellophane) in control conditions. Scale bars are 1 mm. (B) Estimated phytomere length of gametophores grown in red light, measured in figure 11.



Supplemental Figure VI-23 – PpSMXL proteins interactions with PpMAX and among themselves in Y2H experiments Mated yeast carrying the different prey/bait combinations were incubated on selective YNB medium (-His, -Ura, -Trp) containing 0.08% X-Gal, 2% galactose, 1% raffinose, and either 0.1% DMSO or 50µM *rac*GR24 for 72h at 30°C prior to observation.

	DMSO			_	<i>rac</i> GR24 50µМ					
Prey ► Bait ▼	E ²	PPSMXLA	PPSMXLP	PPSMX	C PPSMXLD	E ¹	PPSMXLA	PPSMXLB	PPSMXLC	PPSMXLD
€ ¹	C	6	9	0	0	0	0	Ó	0	0
0KA121.B	0	0	0	0	0	0	0	0	0	0
PP PP	0	0	0	0	0	0	0	0	0	0
PP PP	0	0	0	0	0	0	0	0	0	0
PP KAI2LE	0	0	0	0	0	0	0	0	0	0
PPI	0	0	0	0	0	•	0	0	0	0
PP. G	0	0		0	0	0	0	J	0	0
PPhenolith	0		0	0	0	0	0	Ģ	0	
PP.	0	43	8	C.	Nº 20	6	0	0	0	0
Port	0	0	0	0	0		0	3	•	0
PPK	0	0	3	0	0	0	0	0	0	0
Ppr										

Supplemental Figure VI-24 – PpSMXL proteins interactions with PpKAI2L proteins in Y2H experiments Mated yeast carrying the different prey/bait combinations were incubated on selective YNB medium (-His, -Ura, -Trp) containing 0.08% X-Gal 2% galactose, 1% raffinose, and either 0.1% DMSO or 50µM *rac*GR24 for 72h at 30°C prior to observation.

Up in GFP-PpSMXLC samples compared	d to flag-GFP control samples (37 peptides)	
Majority protein IDs	Phytozome annotation	Predicted process
Pp3c1_21230 (up by GR24 with flag-GFP)	2-CYS PEROXIREDOXIN BAS1, CHLOROPLASTIC	Antioxydation/Redox signaling
Pp3c1_34240	Light-harvesting complex II chlorophyll a/b binding protein 6 (LHCB6)	Photosynthesis (light)
Pp3c10_10490	RHODANESE-LIKE DOMAIN-CONTAINING PROTEIN 4, CHLOROPLASTIC	Photosynthesis (light)
Pp3c10_18330	OUTER ENVELOPE PORE PROTEIN 16-2, CHLOROPLASTIC	POR import, chlorophyll biosynthesis
Pp3c11_12840	65-KDA MICROTUBULE-ASSOCIATED PROTEIN 3-RELATED	Cytoskeleton
Pp3c11_18230	Photosystem I subunit III (psaF)	Photosynthesis (light)
Pp3c11_19220	Founding proteins of phosphatase (TPM), PSII repair	Photoprotection
Pp3c4_13260, Pp3c12_19890, Pp3c12_7030, Pp3c12_7010, Pp3c2_4100,Pp3c19_1860, Pp3c19_1840, Pp3c12_19890, Pp3c22_7400, Pp3c19_3190, Pp3c13_15910, Pp3c15_22720, Pp3c15_22730, Pp3c19_3190, Pp3c12_11880, Pp3c22_540, Pp3c4_11770	Ribulose-bisphosphate carboxylase small chain (rbcS)	Photosynthesis (dark)
Pp3c17_16140, Pp3c17_16070, Pp3c14_26060, Pp3c14_26050, Pp3c14_23440	Solute carrier family 25 (mitochondrial adenine nucleotide translocator)	Mitochondria
Pp3c14 7550	Large subunit ribosomal protein L31e (RP-L31e, RPL31)	Translation
Pp3c15_11070	CHLOROPHYLL A/B BINDING PROTEIN // SUBFAMILY NOT	Photosynthesis (light)
Pp3c15_12410, Pp3c9_5280	26S proteasome regulatory complex, ATPase RPT4	Protein degradation
Pp3c15_13020, Pp3c15_12990	Arachidonate 12-lipoxygenase / 12-lipoxygenase	Oxylipins biosynthesis/OPDA biosynthesis
Pp3c18_4040	Unknown function, moss specific?	
Pp3c18_5520, Pp3c22_17810	Geranylgeranyl diphosphate reductase / Geranylgeranyl reductase	Chlorophyll biosynthesis Kaurene biosynthesis
Pp3c18_8100	Light-harvesting complex II chlorophyll a/b binding protein 1/2 (LHCB1/2)	Photosynthesis (light)
Pp3c19_20900	light-harvesting complex II chlorophyll a/b binding protein 3 (LHCB3)	Photosynthesis (light)
Pp3c19_6730	Actinidain / Actinidin (cysteine protease)	Protein degradation
Pp3c2_34120, Pp3c2_34110	Large subunit ribosomal protein L9 (RP-L9, MRPL9, rpll)	Translation
Pp3c20_15780, Pp3c23_8400	ATP-DEPENDENT ZINC METALLOPROTEASE FTSH 1, CHLOROPLASTIC-RELATED	Photoprotection
Pp3c22_11700 (up by GR24 with flag- GFP)	Thioredoxin-like	Antioxydation/Redox signaling
Pp3c22_5610, Pp3c6_12510	Light-harvesting complex II chlorophyll a/b binding protein 1/2 (LHCB1/2)	Photosynthesis (light)
Pp3c24_12520, Pp3c23_6060	F-type H+-transporting ATPase subunit delta (ATPF1D, atpH)	Cell respiration
Pp3c24_3760, Pp3c23_9380, Pp3c20_19140, Pp3c23_6290	Large subunit ribosomal protein L4e (RP-L4e, RPL4)	Translation
Pp3c26_12240, Pp3c26_12230	Light-harvesting complex II chlorophyll a/b binding protein 5 (LHCB5)	Photosynthesis (light)
Pp3c3_12870	GLUTATHIONE S-TRANSFERASE D2-RELATED	Antioxydation/Redox signaling
Pp3c3_18750	Ribulose-bisphosphate carboxylase small chain (rbcS)	Photosynthesis (dark)
Pp3c3_28050	OUTER ENVELOPE PORE PROTEIN 16-2, CHLOROPLASTIC	POR import, chlorophyll biosynthesis
Pp3c3_3160, Pp3c3_3140, Pp3c13_5060	Enolase (ENO, eno)	Glycolysis
Pp3c4_20230	Phosphoglycerate kinase	Glycolysis
Pp3c4_5690, Pp3c4_5680	Light-harvesting complex II chlorophyll a/b binding protein 4 (LHCB4)	Photosynthesis (light)
Pp3c4_7680	PUTATIVE SEROTONIN TRANSPORTER	Inhibition of auxin response
Pp3c5_7180, Pp3c5_7150, Pp3c5_7110	Light-harvesting complex II chlorophyll a/b binding protein 1/2 (LHCB1/2)	Photosynthesis (light)
Pp3c5_7810, Pp3c6_23221, Pp3c16_15270	F-type H+-transporting ATPase subunit b (ATPF0B, atpF)	Cell respiration
Pp3c6_9630	Similar to tropomyosin actin interacting protein	Cytoskeleton
Pp3c9_3440	CHLOROPHYLL A/B BINDING PROTEIN // SUBFAMILY NOT NAMED	Photosynthesis (light)
Pp3c9_5310, Pp3c22_12960	26S proteasome regulatory complex, ATPase RPT4	Protein degradation
Down in GFP-PpSMXLC samples compa	ared to flag-GFP control samples (1 peptide)	
Pp3c6_6545, Pp3c6_6610, Pp3c6_6560	DIRIGENT PROTEIN 16-RELATED	Specialized metabolism

Supplemental Figure VI-25 – **Detailed interactome of the PpSMXLC protein.** As geranylgeranyl reductase can potentially be involved in both chlorophyll biosynthesis and biosynthesis and of other compounds such as kaurene, it was classified in both the photosynthesis and the specialized metabolism categories.

Bait	FLAG	-GFP	GFP-F	PpSMXLC	None		
	Phytozome + Predicte	e annotation ed process	Phytozom + Predict	e annotation ted process	Phytozome annotation + Predicted process		
		Dehydrin	Pp3c5_20100, Pp3c5_20340, Pp3c6_7400, Pp3c5_20690, Pp3c2_26580, Pp3c6_7500	β tubulin		OUTER ENVELOPE PORE PROTEIN 16- 1, CHLOROPLASTIC	
	Pp3c13 6860	Osmotic stress	Pp3c6_7500, Pp3c9_18440	Cvtoskeleton	Pp3c1 40570	biosvnthesis	
GR24 UP		LRR-tyrosine kinase, ERECTA-like	, poco 10440		Pp3c4_11370, Pp3c22_1860, Pp3c19_6490, Pp3c13_20020	Small subunit ribosomal protein S8e (RP-S8e, RPS8)	
	Pp3c2_22410	Stem cell identity		Small subunit ribosomal protein		Translation	
		Pectinesterase	Pp3c6_8910,			TIP aquaporin	
	Pp3c5_12660	Cell wall	Pp3c5_16070, Pp3c16_9210	Translation	Pp3c23_15870	Redox signaling	
				Glycine cleavage system H protein			
GR24			Pp3c19_11600	Photorespiration			
DOWN			Pp3c9_16100, Pp3c15_16120 Pp3s50_100,	PpSMXLC/D			
			Pp3c9_21600, Pp3c9_21580, Pp3c9_21650	Unknown Pp			

Supplemental Figure VI-26 – Proteins impacted by (±)-GR24.

Supplementary table VI

Cloning of PpSMXL pro	omoters
Primer name	Sequence (recombination/restriction site)
proPpSMXLC-GW-F	CAAGTTTGTACAAAAAAGCAGGCTTCTGAGCTTGCTTGTAGAAAC
proPpSMXLC-GW-R	CCACTTTGTACAAGAAAGCTGGGT GCTCACGTCTATGGTTCTCAC
proPpSMXLD-GW-F	CAAGTTTGTACAAAAAAGCAGGCTGTAATTGTAGATGTGCGTACATCAT
proPpSMXLD-GW-R	CCACTTTGTACAAGAAAGCTGGGTACTCGCGTTCCTATTTCACC
proPpSMXLAF NotI	ATACGTA gcggccgc AGTCTGGCATTGGTCAGAAC
proPpSMXLAR AscI	TACGTAT ggcgcgcc AGTTGCTCACTCTTTCGAATTGT
Cloning of PpSMXL CDS	5 5 5 5 5
Primer name	Sequence (recombination site)
PpSMXLA-GW-START	GGGACAAGTTTGTACAAAAAAGCAGGCTTCATGCGCTCTGGAGCTGCAGC
Posmxla-GW-END	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCCACTGCAGGCAACTTCAA
PDSMXLA-GW-STOP	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCAACTGCAGGCAACTTCAATTTG
PpSMXLB-GW-START	GGGACAAGTTTGTACAAAAAAGCAGGCTTCATGCGCTCTGGGGCAGCAGC
PoSMXLB-GW-END	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCCACTGCAGCCTATTTCGA
PDSMXLB-GW-STOP	CCCCACTACTACAACAACAACCTCCTCAACTCCACCCCACTATTCCCATTTC
PpSMXLC/D-GW-START	GGGACAAGTTTGTACAAAAAAGCAGGCTTCATGCGTAGCGGGGCAAATTC
PoSMXLC-GW-END	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCCCCACACCCACGGTGGACAC
PDSMXLC-GW-STOP	CCCCACCACCTCCACACACACCACCACCACCACCACCACC
PpSMXLD-GW-END	
PpSMXLD-GW-STOP	
PCR-mediated mutagene	asis of PDSMYI CDS
Primer name	
PpSMXLA-ARGRT	
PpSMXLB-ARGRT	
PDSMXLC-ARGKT	
PpSMXLD=ARGKT	
Guide BNAs used for a	PrSMXI. and PrMAX2 mutagenesis
Guide name	Sequence of $crRNA + DAM$
$\alpha RN\Delta - \Delta^{-420R}$	
$\frac{g_{\rm RNA} - \lambda^{+170R}}{2}$	
$\frac{1}{\alpha RN\Delta - \Delta^{+1623R}}$	
$\frac{1}{\alpha RN\Delta - \Delta^{+4083R}}$	
$a B N \Delta - B^{-161F}$	
$\frac{g_{RNA} - B^{+4214R}}{g_{RNA} - B^{+4214R}}$	
GRNA-B ^{+1802F}	
$\frac{g_{\rm RNA} - C^{-207F}}{2}$	
$GRNA - C^{+90F}$	
$\frac{\text{gRNA} - C^{+236R}}{\text{gRNA} - C^{+236R}}$	
$\frac{GRNA - C^{+305R}}{GRNA - C^{+305R}}$	
GRNA-C+339F	
$\frac{GRNA-C^{+1035R}}{GRNA-C^{+1035R}}$	
$\frac{GRNA-C^{+4878R}}{GRNA-C^{+4878R}}$	
apna – D ^{-412F}	
ADNA D	
$\frac{1}{2} \frac{1}{2} \frac{1}$	
adny - Domy 2-19F	
q $PNA = P PMA X 2 + 53F$	CTCAATCCAATCCACACACACACACACACACACACACAC
arna - Pomax2+488F	
aRNA - PpMAX2+516F	
aRNA-POMAX2+591R	
GRNA-POMAX2+1812F	
Guide BNAs used to a	ese integration of constructs at the Dn108 locus
Guide name	Sequence of crRNA + DAM
PD108-CDNA2	
IPIUO-YRNAZ	ATTACTAGTAAAAGCACATAAGG

Chapter VII – PpSMXL proteins regulate growth in diverse contexts

VII-A) PpCCD8-derived compounds (SL) and the PpMAX2-transduced signal (KL) have opposite effects on regeneration

Figure VII-1 – **Effect of** (\pm)-**GR24 and** (+)-**GR24 on regeneration from phyllids.** (A) Dissected phyllids were incubated in the light (long days conditions) in liquid medium containing acetone (control) or (\pm)-**GR24** at different concentrations. Points represent the percentage of regenerating phyllids among 3 in each well of a 96 well-plate (n=16 independent wells). Bars give the median value of replicates. (B) Dissected phyllids were incubated in the dark in liquid medium containing DMSO (control) or (+)-**GR24** at different concentrations. Points represent the number of

chloronema filaments regenerated from each phyllid after 72h (n=22-24 phyllids). Symbols in bold red indicate differences between treated groups and the control group for each genotype (and at each time point in (A)). Symbols in bold blue give differences between mutants and WT in control conditions (Kruskal-Wallis test followed by a Dunn *posthoc* test, $\# 0.1 , <math>* 0.01 \le p < 0.05$, $* 0.001 \le p < 0.01$, *** p < 0.001).

As we noticed opposite effects of (+)-GR24 and (–)-GR24 on the number of *caulonema* filaments grown in the dark, suggesting an opposite role of the two enantiomers on cell division, we tested the effect of these molecules on regeneration. Regeneration can broadly be defined as the development of specialized tissues from a few (or even a single) totipotent cell(s), either pre-existing (stem cell(s)) or obtained by dedifferentiation of (a) specialized cell(s) (Birnbaum and Alvarado, 2008). (\pm)-GR24 effect as an inhibitor of *protonema* regeneration from protoplasts has already been shown (Lopez-Obando et al., 2018), however the role of separate enantiomers had yet to be clarified. To have a clearer view of the expected effects of endogenous compounds mimicked by (+)-GR24 (SL) and (–)-GR24 (mainly KL) on regeneration, we also included the *Ppccd8* and the *Ppmax2-1* mutants in our tests, the former not producing SL and the latter putatively displaying a KL-insensitive phenotype. We started by carrying out regeneration assays on dissected phyllids, as we expected a very poor regeneration of protoplasts in *Ppmax2-1* and therefore potential masking of (\pm)-GR24 enantiomers effects (figure VII-1).

In this new experimental setup, the repressive effect of (\pm) -GR24 on regeneration was replicated as seen previously on protoplasts, as regeneration of WT phyllids was affected even by only 30 nM (\pm) -GR24, at 48h and 96h (figure VII-1A). Additionally, concordantly to the more rapid germination of *Ppccd8* spores (Proust et al., 2011), this SL-deficient mutant has a significantly higher regeneration ability than WT in the absence of (\pm) -GR24 treatment. On the contrary, the *Ppmax2-1* mutant's regeneration is dramatically decreased compared to WT. Regeneration abilities of these two mutants in the absence of (\pm) -GR24 treatment hint at PpCCD8-derived compounds (putative SL) having a negative impact on regeneration, while the PpMAX2-transduced signal(s) (putative KL) would have a positive role in regeneration. Interestingly, by testing three concentrations of (\pm) -GR24, we noticed that the effect was not clearly dosedependent except for *Ppmax2-1*, which was particularly blatant at the 72h time-point for WT. We thought that this phenomenon could stem from the more limited effect of the (-)-GR24 enantiomer present in the mixture, which would become more visible when high concentrations of (\pm) -GR24 are used. Moreover, another surprising observation was that *Ppccd8* regeneration did not appear more affected by (\pm) -GR24 treatments than WT, unlike in *caulonema* growth assays in the dark (see chapters IV and VI).

Reasoning that this non dose-dependent effect could be explained by the use of the racemic mixture, we carried out a similar experiment using (+)-GR24 (figure VII-1B). Furthermore, we let phyllids regenerate in the dark, to separate the effects of light from that of (+)-GR24 addition. In this second experiment, we could observe a significant negative effect of 300 nM (+)-GR24 on WT regeneration, which suggests that phyllids are more responsive to (+)-GR24 in the dark (in the light, the effect of (\pm)-GR24) was not significant even at 3 μ M). Here again, regeneration of *Ppccd8* in control conditions was higher than that of WT, while *Ppmax2-1* regeneration was almost null. *Ppccd8* responded better than WT, while *Ppmax2-1* response was only revealed by a tendency here, owing to its very low level of regeneration that (+)-GR24 mimics PpCCD8-derived compounds and has a dose-dependent negative impact on regeneration from phyllids.

Figure VII-2 – **Effect of** (+)-**GR24 and** (-)-**GR24 on protoplasts regeneration in the light.** (A) Effect of (+)-GR24, at 3, 30 and 300 nM. Control is DMSO 0.3%. Points represent the percentage of regenerating protoplasts in a drop (n=27 drops across 3 plates), 72h after the start of cultivation. (B) Effect of (-)-GR24 at the same concentrations. Control is DMSO 0.3%. Points represent the percentage of regenerating protoplasts in a drop (n=24 drops across 4 plates), 72h after the start of cultivation. (C) and (D) Follow up of the experiments respectively presented in (A) and (B), 6 days after the start of cultivation. (A) (B) (C) and (D) Bars indicate median values. Bold red symbols indicate statistical significance of comparisons of GR24 treated samples to control samples within genotype (Kruskal-Wallis test followed by a Dunn *post-hoc* test, * $0.01 \le p < 0.05$, ** $0.001 \le p < 0.01$, *** p < 0.001). Blue bold symbols give the statistical significance of comparisons of mutants control groups to WT (Mann-Whitney test, * $0.01 \le p < 0.05$, *** p < 0.001). Note the difference in overall regeneration levels between panels A and B, which reveals a difference in the quality of protoplasts preparation between the two experiments.

As we have seen in *caulonema* growth assays that the effects of (-)-GR24 are often quite discrete compared to those of (+)-GR24, we switched to more sensitive protoplasts regeneration assays to assess the effects of separate (\pm)-GR24 enantiomers (Lopez-Obando et al., 2018) (figure VII-2). This change also enabled us to keep using low concentrations of enantiomers (3, 30 and 300 nM). Moreover, by slightly modifying our previous protoplasts preparation protocol, we could obtain enough robust protoplasts for *Ppmax2-1*.

(+)-GR24 had a dose-dependent repressive effect on regeneration from protoplasts at 72 hours, both in WT and *Ppccd8* (figure VII-2A). Strangely, unlike in *caulonema* growth tests, while *Ppccd8* regenerated better than WT in the absence of treatment, it did not respond more strongly to (+)-GR24, similarly to what was noted in phyllids regeneration tests carried out in the light (figure VII-1A). On the contrary, a higher dose was necessary to induce a significant drop in regeneration. This could be explained by the different light conditions. Indeed, both in transcriptional response and *caulonema* growth assays, which are monitored in the dark, *Ppccd8* has a clearer response to (+)-GR24 compared to WT. Hence, we can hypothesize that the well-documented positive impact of light on protoplast regeneration (Jenkins and Cove, 1983) is somehow more intense in *Ppccd8* than in WT and thus that PpCCD8-derived compounds interfere with this response to light in WT. (-)-GR24 also had a dose-dependent effect on protoplasts regeneration in WT (figure VII-2B and D). As we hypothesized, this effect was opposite to that of (+)-GR24 and its amplitude was lower, possibly because (-)-GR24 also stimulate the SL pathway (see chapters IV and VI). Response of *Ppmax2-1* was impossible to assess after only 72 hours of regeneration (figure VII-1A and B). Therefore, we observed the protoplasts again after an additional 72 hours (6 days in total, figure VII-2C and D). At this later time point, the opposite response of WT to both enantiomers was still evident, and we could note that *Ppmax2-1* does respond to (+)-GR24 (figure VII-2C) but this response is decreased compared to WT. On the other hand, *Ppmax2-1* does not respond to (-)-GR24 (figure VII-2D).

The dose-dependent response to (\pm) -GR24 enantiomers in protoplasts regeneration assays apparently validates our previous hypothesis that, (\pm) -GR24 has a non-dose-dependent effect in phyllids regeneration assays because of the influence of the (-)-GR24 enantiomer. However, this phenomenon could also stem from a bias specific to our phyllids regeneration assays, since we previously noted a dose-dependent effect of (\pm) -GR24 in protoplasts assays (Lopez-Obando et al., 2018). Hence, we had to fine-tune the experimental setup for phyllids regeneration assays, notably carrying them out in the dark where response to (+)-GR24 become dose-dependent (see methods chapter in the annex). Until now, phyllids assays could not reveal the effect of (-)-GR24, which suggests that these assays are better suited to study the effect of SL mimics rather than that of KL mimics. Although regeneration from phyllids and protoplasts can be broken down into two main processes (dedifferentiation followed by cell division) and our experiments cannot really determine which out of the two is impacted by treatment with (\pm) -GR24 enantiomers, we can infer that at least the cell division process is affected. Indeed, cell division is specifically affected in opposite ways by the two enantiomers in dark grown *caulonema* (see chapters IV and VI, as deduced from the number of *caulonema* filaments). Moreover, oppositely modified cell division rates in the *Ppccd8* and *Ppmax2-1* mutants could also explain their respective *protonema* growth in control long-days conditions.

These experiments moreover underline the major impact of light on response to (\pm) -GR24 enantiomers. Indeed, contrary to what we thought until now based on tests using the (\pm) -GR24 mixture and from testing the enantiomers on *caulonema* growth in the dark, (-)-GR24 does have a dose-dependent effect on WT, which is opposite to the effect of (+)-GR24. The effect of the (-)-GR24 enantiomer could be clearer in light conditions, which might extend to the effect of endogenous KL and of karrikins. Thus, it would be interesting to check whether *protonema* extension in control long-days conditions is affected in response to (-)-GR24 and KAR₂ addition, in a dose-dependent manner.

VII-B) Higher order *Ppsmxl* loss of function mutants have a decreased regeneration ability

As both SL and KL signaling appeared to play a role in regeneration, we investigated the ability of *Ppsmxl* mutants phyllids to regenerate in the light, in the absence of GR24 treatments. *Ppsmxlab* double mutants tended to regenerate more slowly than WT, whereas *Ppsmxlcd* double mutants appeared to regenerate more quickly (figure VII-3). This tendency for *Ppsmxlcd* was supported by our previous observations that, in the process of obtaining *Ppsmxlc/d* mutants, WT protoplasts transformed with guide RNAs targeting *PpSMXLC* and *PpSMXLD* regenerated better than untransformed controls. Higher order *Ppsmxl* mutants were much more affected than double mutants and their regenerative ability was decreased. This observation is quite difficult to explain, as higher order mutants' phenotype does not correspond to an additive effect of the mutation of clades A/B and C/D. However, since we have established before that these higher order mutants have a highly restricted *protonema* growth, certainly linked to a senescence phenotype, it is possible that this senescence interferes with the regeneration process here.

The observation that both clades have apparently opposite roles in regeneration is quite surprising considering our previous finding that both clades similarly act as negative actors in the PpMAX2-dependent pathway. However, since both clades are not preferentially expressed in the same tissues and life-stages, the effects of *PpSMXL* function loss we report in this experiment might not hold biological significance in physiological growth. Still, this finding does raise questions about potential different interactors between the two clades, that could explain this opposite outcome on regeneration. The regeneration ability of *Ppsmxl* simple mutants should also be investigated to clarify the role of each *PpSMXL* gene in the context of regeneration. Protoplasts regeneration of *Ppsmxl* mutants will also be assessed, moreover in response to (\pm) -GR24 enantiomers, as we found that this type of assay is more sensitive than phyllids regeneration.

Figure VII-3 – **Regeneration from phyllids in the light is affected in** *Ppsmxl* **mutants.** Points represent the percentage of regenerating phyllids out of 16 from a given plant (n=3 individual plants). Bars are the median of the 3 replicates. Symbols in bold red indicate the statistical significance of the regeneration difference relative to WT at each time point (Kruskal-Wallis test followed by a Dunn *post-hoc* test, $\# 0.1 , <math>* 0.01 \le p < 0.05$).

VII-C) PpSMXL are not *bona fide* heat-shock proteins but they play an important role in temperaturemediated growth regulation

Since we found that PpSMXL proteins are not repressors in the SL signaling pathway, we wanted to clarify the molecular function of these proteins in development. Notably, we sought whether they have similar properties as more distant ClpATPases such as ClpB/HSP101 (see chapter V). First, we investigated the profile of *PpSMXL* genes expression when WT *P. patens* was subjected to 3 hour-long incubations at different temperatures. Two different experiments were carried out, to extend the range of temperatures examined. In a preliminary *in silico* search, we found that *PpSMXL* promoter sequences and 5'UTR regions contain several heat-shock elements and/or similar sequences (Supplemental Figure VII-1), which seem broadly conserved between *P. patens* and Angiosperms (Schöffl et al., 1984; Elzanati et al., 2020). Hence, we thought that expression *PpSMXL* might be induced by heat. However, we observed that none of the four *PpSMXL* display the characteristic profile of HSPs e.g. a steep increase of expression at high temperature (see Supplemental Figure VII-2 for the profile of *PpHSP19*). Instead, we discovered that, within A/B and C/D clades, each gene seems to have a different reaction to temperature: *PpSMXLA* is induced by heat (42°C) and repressed by cold (8°C and 15°C), *PpSMXLB* is repressed by heat (42°C) and seemingly also repressed by cold (15°C) (figure VII-4). On the other hand, *PpSMXLC* expression is induced by cold (8°C) but does not seem sensitive to heat, and *PpSMXLD* is the most temperature-responsive gene out of the four *PpSMXLL* it is repressed by heat (42°C, and non-significantly at 37°C) and induced by cold (4°C, and non-significantly at 8°C).

In order to determine the physiological implications of these gene expression profiles, we investigated the growth of *Ppsmxl* mutants in cold stress conditions (at 10° C) and after a one-week recovery period (at 25° C) (our experimental setup is illustrated in panel A of figure VII-5). Our starting basis was that tolerance to the cold treatment would be reflected by a moderate decrease in growth during the cold period and a rapid resumption of growth after return to control conditions.

Figure VII-4 – **Transcript levels of** *PpSMXL* **genes are affected by temperature.** Transcript levels of the four *PpSMXL* genes are given relative to the two reference genes PpElig2 (*Pp3c14_21480*) and PpAPT (*Pp3c8_16590*). RTqPCR data used for the analysis was extracted from 6 biological replicates, each with 2 technical repeats (in a given experiment). Each point represents the mean of these 2 technical repeats. Bars indicate median values. Statistical significance scores of differences relative to 25°C are indicated by bold symbols for each gene (Kruskal-Wallis test followed by a Dunn post-hoc test, $\# 0.1 , <math>* 0.01 \le p < 0.05$, $* 0.001 \le p < 0.01$, *** p < 0.001).

The *Ppccd8* mutant had a higher initial loss of growth (after 1 week in cold conditions) but then an equivalent relative limitation of growth as WT during the second week. Moreover, its recovery was better than WT (as shown by the slope of the second part of the curve in figure VII-5, and table VII-1). This behavior implies that PpCCD8-derived compounds (and by extension SL) initially repress growth limitation in reaction to cold and limit recovery but do not impact the acclimation ability. Therefore, PpCCD8-derived compounds tend to keep growth stable when temperature changes, which could be a form of tolerance to cold. On the other hand, growth of *Ppmax2-1* was only slightly more limited than WT initially but decreased much more after the second week in cold conditions. However, recovery seemed equivalent to WT. Together, these observations suggest that PpMAX2 limits decreases in growth in the second part of our cold treatment. Thus, PpMAX2 could play a role in acclimation to cold.

As suggested by the four genes' expression profiles, simple mutants of the same clade did not display the same response to the cold-stress treatment (figure VII-5B and Supplemental Figure VII-3). Starting with clade A/B, the *Ppsmxla5* simple mutant growth was less restricted by cold compared to WT during the first week, however it was more affected during the second week. Furthermore, its recovery slope was less steep than for WT. Hence, it seems that *PpSMXLA* rapidly limits growth in cold conditions and is necessary for acclimation to cold and then for quick recovery when stress is alleviated. The simple $Ppsmxl\Delta b7$ mutant is only slightly more impacted than WT by cold. However, during its recovery, slope is comparable to that of *Ppsmxla5* (see percentages given in table VII-1). Therefore, while *PpSMXLB* could play a minor opposite role to *PpSMXLA* in cold-triggered limitation of growth, it most importantly plays an equivalent role in promotion of recovery. The double $Ppsmxla8\Delta b6$ mutant indeed was more alike Ppsmxla5in its reaction to cold stress, underlining the predominant effect of *PpSMXLA*. Moreover, recovery of the double mutant was completely abolished, illustrating an additive action of *PpSMXLA* and *PpSMXLB* during this process. It is surprising to observe such a major effect of *PpSMXLA* loss of function by itself. Indeed, since this protein is virtually not expressed except in spores, we initially thought that this gene might be in the process of pseudogenization. However, PpSMXLA function might be very relevant in particular cases such as response to stresses. While we did not investigate yet the role of *PpSMXL* genes in tolerance to desiccation, it would be very interesting to do it soon, as all four *PpSMXL* are highly expressed in spores which are desiccation-resistant cells.

Figure VII-5 – *Ppsmxl* mutations in the same clade have a different impact on reaction to cold stress and subsequent recovery. (A) Scheme of the experimental setup. All plants (n=6 plates x 9 plants = 54) were grown at 25°C in for 2 weeks. Then half of the plants (n=27 plants, 3 plates) were transferred at 10°C, in the same lighting and hygrometry conditions, while the other half remained at 25°C as a control. After 2 weeks at 10°C, cold-stressed plants were returned to 25°C for 1 week. Diameter of each plant was assessed just before transfer, then in the middle of the cold stress period, at the end of the cold stress period and finally at the end of the experiment after 1 week of recovery at 25°C. (B) Mean relative decrease of growth in the cold-stressed groups compared to the control group, for each

genotype and at each time point (loss is expressed in percentage and was estimated as follows: (diameter_{10°C}-diameter_{25°C})/diameter_{25°C}).

When we focused on the C/D clade, we found that the *Ppsmxl* $\Delta c4$ simple mutant growth was initially more decreased by cold than for WT, but this mutant eventually resumed growth (see the 2 weeks timepoint). This suggests that *PpSMXLC* function is important to maintain growth during cold stress, but that role becomes reversed under long-term cold stress. This suggests that acclimation to cold is repressed by PpSMXLC in WT plants. The recovery of *Ppsmxl* $\Delta c4$ was only slightly decreased compared to WT, so *PpSMXLC* might not play an important role during this step. As for the *Ppsmxld1* mutant, its reaction to cold stress was almost identical to that of WT, but recovery was completely abolished. The capital function of *PpSMXLD* for recovery to cold-stress fits with the induction of this gene expression by cold. We could hypothesize that the PpSMXLD protein accumulates in cold-stressed plants and acts as a chaperone to either protect proteins necessary for recovery or maybe prevents the action/accumulation of defective proteins that could hamper recovery. The *Ppsmxl* $\Delta c7\Delta d4$ double mutant was initially more affected by cold than WT, even more than *Ppsmxl* $\Delta c7\Delta d4$ recovered better than WT and both simple mutants of the C/D clade. Therefore, the simultaneous loss of *PpSMXLC* function alleviates the dramatic effect of *PpSMXLD* loss of function on recovery.

Strikingly, the phenotype of $Ppsmxl\Delta c7\Delta d4$ is here again akin to the SL deficient Ppccd8 mutant, suggesting the molecular processes disturbed in the two mutants are the same. This observation is very puzzling because we have seen that loss of PpCCD8 function results in increased transcript levels of PpSMXLC and PpSMXLD (chapter VI). Alternatively, similar reaction to cold of Ppccd8 and $Ppsmxl\Delta c7\Delta d4$ could be explained by the prevalence of *protonema* over gametophores in these two mutants. It is also worthy of note that Ppsmxl mutants and Ppmax2-1 do not display opposite responses to cold, which could imply that in these conditions PpSMXL proteins exert PpMAX2-independent roles.

	Relative loss week 1	Relative loss week 2	Relative gain recovery
WT	-28%	-17%	10%
Ppccd8	-37%	-17%	17%
Ppmax2-1	-33%	-20%	13%
$Ppsmxla8\Delta b6$	-17%	-21%	0%
$Ppsmxl\Delta c7 \Delta d4$	-39%	-15%	17%
Ppsmxla5	-15%	-22%	6%
$Ppsmxl\Delta b7$	-33%	-16%	5%
$Ppsmxl\Delta c4$	-35%	-11%	8%
Ppsmxld1	-26%	-18%	-1%

Table VII-1 – **Comparison of growth in response to cold.** Percentages of relative growth loss/gain were calculated as the difference of growth between two successive time-points. These values are proportional to the slopes of curves given in figure VII-5. Differences with WT equal or superior to 5% are deemed significant: green = higher loss/gain than WT, red = lower loss/gain than WT.

VII-D) PpSMXL proteins play important roles in regeneration and stress tolerance

We found that loss of *Ppsmxl* function, and furthermore disturbance of either the SL pathway (in *Ppccd8*) or the KL pathway (in *Ppmax2-1*), has major implications on regeneration and growth regulation in response to cold stress. However, while the effects of the two pathways are clearly opposite in the case of regeneration, their respective roles in response to cold is more ambiguous.

In order to clarify these findings, we must replicate the cold stress experiment (notably including the triple *Ppsmxla1b1d1* mutant) and to devise similar experiments to assess the effect of *Ppsmxl* mutations in the context of heatstress and in the context of dehydration. As for regeneration assays, we ought to carry them out on simple *Ppsmxl* mutants in order to determine the role of each *PpSMXL* gene in this process. As the cold stress experiment showed, these genes might have different functions even within the same clade. Given the complex impact of combinations of *Ppsmxl* mutations, one possible explanation is that the PpSMXL proteins might be able to interact together, maybe forming oligomeric complexes as other Clp-ATPases (see chapter V). We can imagine that the different complexes have slightly different function, putatively by interacting with different sets of target proteins. Also, it is worthy of note that these functions of PpSMXL proteins are exerted when there is no KL signal transduction, and reinforced in the presence of SL in the case of PpSMXLC/D. Hence, SL can be read as stress hormones in *P. patens* (as was suggested already in Angiosperms), while KL would be a growth promoting signal limiting stress response.

Interestingly, we show here that *PpSMXLC* seems to have a divergent role compared to the other three *PpSMXL* genes, which is coherent with our previous concluding statements in chapter VI. While its loss has the most predominant effect on growth in control conditions (see chapter VI), it is the less transcriptionally regulated by temperature and the one with the less predominant effect on cold tolerance (see figure VII-5). Hence, the PpSMXLC protein might have a different scope of action compared to the other PpSMXL proteins. This specificity could be explained by PpSMXLC having specific interactors, replication of our GFP-trap experiment (see figure VI-14 and supplemental figures VI-25 and VI-26) for PpSMXLA, B and D could reveal such discrepancy.

Methods specific to chapter VII:

Phyllids regeneration assay. In the experiment presented in figure VII-1A, a variation of the protocol detailed in our Methods chapter (see annex 1) was employed, where phyllids were regenerated in long days conditions instead of in the dark. For the experiment presented in figure VII-1B, this protocol was followed without modifications.

Protoplasts regeneration assay. A variation of the protocol used by (Lopez-Obando et al., 2018) was carried out using WT, *Ppccd8* and *Ppmax2-1* protoplasts. Protoplasts were isolated after a 30 minute-long 1% driselase digestion at 25°C and 3 subsequent washing steps in 8.5% mannitol. After a night of recovering in PpNH₄ supplemented with mannitol, glucose and CaCl₂ (300 mg/L) in the dark, protoplasts solutions are mixed with an equal volume of a 2% alginate 8.5% mannitol solution. Before polymerization occurs, ~50 µL drops (containing ~100 protoplasts) of this mix are deposited on solid PpNH₄ mannitol CaCl₂ medium, supplemented with DMSO (0.3% final concentration) or (\pm)-GR24 enantiomers, overlaid with cellophane. Plates are incubated in long-days control conditions until observations 72 hours later.

Cold stress assay. Small fragments of *protonema* were grown in control conditions (16h day/8h of night, ~90µmol m⁻² s⁻¹ white light, ~55% hygrometry, 25°C during the day and 23°C at night) for two weeks, on low nitrogen content medium (PpNO₃) without underlying cellophane (initiation period). Half of the plants were transferred to a growth cabinet with the same growth conditions except for the temperature (10°C in days and nights) and the half remained at 25°C, for two more weeks (stress period). Finally, all plants were grown at 25°C for an additional week (recovery period). Diameters were monitored at 4 timepoints: at the end of the initiation period, after one week of stress, at the end of the stress period, and finally at the end of the recovery period. The effect of stress and recovery was assessed for each genotype as the normalized difference between the mean diameter of plants incubated at 10°C and the mean diameter of plants kept at 25°C throughout the experiment (control groups).
	Promoter + upstream gene 3'/5' untranslated						5' untranslated region					
	region (for A and B only)											
	HSE	Inr	CCAAT	G	TATA	GC	HSE	Inr	CCAAT	G	TATA	GC
			box	box	box	box			box	box	box	box
PpSMXLA	2 (+5 HSE- like)	1	1	0	No	0	13 HSE- like	10	1	0	No	0
PpSMXLB	2 (+22 HSE- like)	0	1	1	No	2	20 HSE- like	5	0	2	No	1
PpSMXLC	7 HSE- like	4	3	0	Yes	2	1 (+15 HSE- like)	5	0	1	No	3
PpSMXLD	17 HSE- like	5	0	2	Yes	1	3 (+14 HSE- like)	3	3	0	No	0

Supplemental Figure VII-1 – **Putative transcription activating elements in** *PpSMXL* **promoters and 5'UTRs.** *PpSMXL* promoter sequences (relative to the Start of translation codon ATG) extends in the following ranges: from - 2617bp to -1978bp for *PpSMXLA* (begins where Pp3c2_14210V3.1 3'UTR ends), from -3106bp to -2086bp for *PpSMXLB* (begins where Pp3c1_23558V3.1 3'UTR ends), from -3768bp to -2162bp for *PpSMXLC*, and from -3460bp to -1997bp for *PpSMXLD*. Sequences enabling binding of general transcription factors were also searched for, such as CCAAT boxes, GC boxes (GGGCGG consensus sequence), Initiator (Inr) motifs and TATA boxes. Inr sequences correspond to the exact consensus YTCANTYY sequence (where Y stands for C or T and N for any nucleotide), according to Nakamura et al., 2002. Putative TATA boxes were searched for in ~50pb upstream of the most upstream starting transcrit found on Phytozome, or anywhere in the 5'UTR (looking for possible alternative TSS). Enhancer cisacting elements such as G-Boxes (CACGTG consensus) and HSE were also found. HSE (heat shock elements) have been searched for using the core consensus sequence GAANNTTC (according to Schöffl et al., 1984). Motifs corresponding to this consensus with only one mismatch are taken as HSE-likes.



Supplemental Figure VII-2 – Relative expression of temperature sensitive genes in different temperatures. Transcript levels of published heat-induced (Pp3c19_10440, Elzanati et al., 2020) and cold-induced (Pp3c9_5910 and Pp3c25_1480, Beike et al., 2015) genes are given relative to the two reference genes PpElig2 (Pp3c14_21480) and PpAPT (Pp3c8_16590). RT-qPCR data used for the analysis was extracted from 6 biological replicates, each with 2 technical repeats (in a given experiment). Each point represents the mean of these 2 technical repeats. Bars indicate median values. Statistical significance scores of differences relative to 25° C are indicated by bold symbols for each gene (Kruskal-Wallis test followed by a Dunn post-hoc test, # $0.1 , * <math>0.01 \le p < 0.05$, ** $0.001 \le p < 0.01$, *** p < 0.001).



Supplemental Figure VII-3 – **Data used to assess decrease of growth in cold conditions in figure VII-5.** Distributions of diameters of plants maintained at 25°C are shown as solid-colored boxplots. Those of cold-stressed plants are shown as hatched boxplots. Times of measurements correspond to those indicated by small cameras in figure VII-5, panel A.

Chapter VIII – Global Discussion



VIII-A) Current model of SL and MAX2-dependent signaling in P. patens

Figure VIII-1 – PpSMXL proteins are major regulators of the *protonema*/gametophores growth balance, integrating KL and SL signals.

According to results and considerations presented in previous chapters, the model presented above in figure VIII-1 could explain how responses to PpCCD8-derived compounds (SL) and to KL (via PpMAX2) are linked in *Physcomitrium patens*. Green arrows denote positive effects while red rounded end pointers denote negative effects. Intensity of coloration reveals the level of activity of the pathway or of given regulations. Dotted lines show effects that have not yet been proven experimentally, plain lines show effects backed up by evidences given in chapters IV, VI and VII. For better clarity, transcriptional regulation of genes acting in these pathways are not included. Our hypothesis that the SL pathway acts by stabilizing/activating PpSMXLC/D is illustrated by the dotted green arrows. In the upper panel, balance between the SL and KL pathways leads to a WT phenotype. In *Ppmax2* mutants, the PpKAI2L(A-E)-PpMAX2 pathway is shut down, leading to a collective increase of PpSMXL protein levels/activity. Since PpSMXL limit *protonema* growth in favor of gametophore development, these mutants prematurely develop gametophores. In the

Ppccd8 mutant, the PpKAI2L(JGM)-dependent pathway is off (in the absence of exogenous SL or mimics). Thus, PpSMXLC/D are not as stabilized/activated as in WT and PpSMXL levels/activity is globally decreased. This leads a de-repression on protonema growth and therefore *Ppccd8* develops extended protonema. In *Ppsmxlabd* triple mutants, the effect of SL is lost and the putative specific function of PpSMXLC in senescence promotion is not downregulated by the other PpSMXL anymore. Senescence eventually even overruns the effects of de-repression on *protonema* growth. In *Ppsmxl* simple mutants, no major developmental phenotype like in *Ppccd8* is observed because PpSMXL proteins play a redundant role in preserving the protonema/gametophores balance. Nonetheless, the slightly more affected *Ppsmxlc* mutants reveal that PpSMXLC has a prominent effect on this developmental balance. In the double *Ppsmxlcd* mutants, the stabilizing/inductive effect by SL signaling is lost. Subsequent decrease of PpSMXLC/D activity leads to a de-repression on *protonema* growth, replicating the phenotype of *Ppccd8*. The slight effect of PpSMXLA/B cannot ensure sufficient repression of *protonema* growth in the absence of PpSMXLC/D. Consequently, in the double *Ppsmxlab* mutants, no major effect is noted on the protonema/gametophores balance, however other phenotypes might be disturbed (notably spore germination given *PpSMXLA/B* expression patterns). Treatment with the unspecific (-)-GR24 enantiomer in *Ppmax2* further limits *protonema* growth because it can only be perceived through the SL signaling pathway, which leads to even higher PpSMXLC/D levels/activity. Hence, (-)-GR24 has an opposite effect on caulonema number on WT and Ppmax2-1. In Ppccd8, PpKAI2L(JGM) receptors are not occupied by endogenous SL, thus (-)-GR24 can easily "spill out" on the SL signaling pathway, here again leading to an opposite effect on *caulonema* number compared to WT.

VIII-B) Current views on SL signaling evolution

Specific receptors (KAI2/DDK) and SMXL proteins associated with either the KAR/KL pathway or the SL pathway seem to be an Angiosperms innovation (Bythell-Douglas et al., 2017; Walker et al., 2019). Indeed, the canonical D14 SL receptor appeared after the emergence of seed plants, and the SMXL was only recently expanded in Angiosperms. This raises several questions: (1) Did ancestral land plants perceive and transduce the SL signal? (and if yes, how?); (2) How can SL perception and transduce occur in extant SL-sensitive non-Angiosperms?

It is important to note that, for the moment, SL sensitivity outside of Angiosperms is only documented in *P. patens*, albeit SL biosynthesis is shared by all land plants and even some algae. Indeed, while other studies explored the effects of exogenous (\pm) -GR24 on development in other Bryophytes or even in algae, the use of the racemic mixture does not permit to known if it is actually an effect of SL that is observed or a stimulation of the KL pathway (Delaux et al., 2012).

In the moss *P. patens*, two main, non-exclusive, hypotheses can explain SL sensitivity: (1) Multi-functionality of ancestral DDK and SMXL proteins, which has been conserved in *P. patens* lineage; (2) Convergent evolution: DDK and SMXL homologs were independently recruited to act in SL signaling in *P. patens* lineage and Angiosperms.

Previous studies have already supported the convergent evolution hypothesis in the case of KAI2/DDK receptors (Lopez-Obando et al., 2016a; Bythell-Douglas et al., 2017). Indeed, in bryopsids mosses, the expansion of the KAI2L protein family following WGD would have been followed by a neofunctionalization event much like in parasitic

Angiosperms (Conn et al., 2015; Bythell-Douglas et al., 2017), thereby giving rise to KAI2L receptors with switched ligand specificity towards SL, at least for *P. patens*. This was confirmed by our findings, presented herein in chapter IV.

Following the hypothesis of convergent evolution, the presence of an additional SMXL clade in bryopsids mosses could likewise hint at a neo-functionalization event, enabling some SMXL to act in SL signaling while retaining the ancestral ability to interact with MAX2. Following this hypothesis, SL signaling in *P. patens* would follow the same core pathway as in Angiosperms. This hypothesis of SMXL convergent evolution was recently challenged by the discovery that the MAX2 homolog of *P. patens* is not necessary for response to SL (Lopez-Obando et al., 2018). While it cannot be excluded that another F-box protein can undertake the role of MAX2 in SL signaling in *P. patens*, it suggests that either the ancestral role of MAX2 was not in SL signaling, or that MAX2 lost this function in *P. patens* evolutive history.

However, as phylogenetic studies conclude that KAI2 and SMAX1 functions are ancestral in the respective KAI2/DDK and SMXL protein families, and as the *MAX2* is conserved as a single copy gene in land plants, the most supported hypothesis is that the ancestral KAI2-MAX2-SMAX1 pathway was involved in KL signaling, and that this function was conserved in *P. patens* lineage. Supporting this hypothesis, we have shown in chapter VI that SMXL proteins of *P. patens* are indeed repressors in the PpKAI2L-PpMAX2 pathway (putative ancestral KL pathway). While the function of PpSMXLC/D is also necessary for SL response, they do not ensure the same function in SL signaling as in Angiosperms: they are not repressors of response to SL. Rather, our results suggest that PpSMXLC/D have a positive role in the response to SL. Our current hypothesis explaining the transduction of the SL signal in *P. patens* is that it acts by inhibiting the KL pathway, possibly by stabilizing PpSMXLC/D proteins (Figure VIII-1). However, additional experiments are needed to provide clear evidence supporting this hypothesis.

Hence, in *P. patens* lineage only the DDK receptor was recruited in evolutive convergence with Angiosperms, while MAX2 and SMXL were only recruited in Angiosperms. It would be interesting to investigate the SL response ability and SL signaling in other mosses to determine when DDK recruitment occurred and if SL response via SMXL proteins is a common mechanism in mosses. Also, other Bryophytes with sequenced genomes, such as *Marchantia polymorpha* (Bowman et al., 2017) (*M. paleacea* would be more relevant, as *M. polymorpha* likely lost the ability for SL biosynthesis) and *Anthoceros agrestis* (Li et al., 2020a), should be studied to determine whether SL response ability is actually specific to mosses amongst Bryophytes.

VIII-C) About the role(s) of SL in extant mosses

A remaining question is why the ability to respond to SL was selected along *P. patens* evolution: What was the significant advantage for fitness that response to SL granted to *P. patens* ancestors?

In the past decade, it has been suggested that SL are used by bryopsids mosses as Quorum Sensing (QS) molecules instead of hormones *per se*, and that the ancestral function of SL was communication with the rhizosphere microbiota (AMF) (Proust et al., 2011; Walker et al., 2019). We can oppose to this view the observation that SL in *P. patens* repress their own biosynthesis (Proust et al., 2011) (as in Angiosperms), while for QS signals a positive feedback

mechanism enables accumulation of the molecule (Waters and Bassler, 2005). Therefore, we could argue that SL in the medium act more as a negative allelopathic signal.

Still, as we have seen in chapters IV and VI, as well as was already suggested by previous studies in *P. patens* (Proust et al., 2011; Lopez-Obando et al., 2018), SL (or rather PpCCD8 derived compounds) actually have an hormonal activity in this species. Indeed, while SL are liberated into the medium where they affect other individuals of the same species (Hoffmann et al., 2014), they also hold an endogenous effect. Indeed, the *Ppccd8* SL deficient mutant displays its extension phenotype even when grown alone, which shouldn't be the case if SL were only relevant in the environment.

Along its evolutive history, *P. patens* lost the SL biosynthesis gene *MAX1* (Walker et al., 2019), most probably involved in the specific generation of canonical SL. Canonical SL have been shown to be the major actors in the positive role of SL towards AMF symbiosis, which might be linked to their increased stability relative to non-canonical SL, likely making them slightly more persistent in the soil (Yoneyama et al., 2018b). Thus, the loss of this canonical SL biosynthesis gene seems coherent with the lack of AMS ability in this moss. However, loss of *MAX1* is not common to all mosses (Walker et al., 2019). Thus, considering *MAX1* as a purely symbiosis associated gene might be far-fetched. Indeed, when MAX1 activity is not followed up by another enzyme (see chapter III), non-canonical SL might be the end-product of SL biosynthesis, as shown in Arabidopsis and hemp (Abe et al., 2014; Huet et al., 2020) (see chapter IV).

It was suggested that two main drivers can explain the loss of AMS and associated genes: a nutrient rich ecological niche (makes symbiosis too costly for a meager usefulness), or a high pathogen pressure (makes intracellular symbiosis risky as it can be diverted by pathogenic micro-organisms) (Radhakrishnan et al., 2020). We cannot know whether any of these happened in *P. patens* evolutive history. The loss of *CCD7* and *CCD8* in *P. patens* evolutive history would have been prevented by the emergence of a new pathway responding to SL, after neofunctionalization of KAI2L proteins. Furthermore, SL biosynthesis confers resistance against phytopathogenic fungi (Decker et al., 2017), so it is tempting to assume that *CCD7* and *CCD8* were kept because of a selection pressure from pathogenic origin.

Given these considerations, we can hypothesize that the ability to excrete SL into the medium is ancestral, as it is needed for inducing symbiosis, and was conserved in *P. patens* lineage with another purpose: co-regulation of growth across a local population. That might permit this species to limit intraspecific competition and instead redirect growth (*protonema* extension) towards patches where this species is absent, thus increasing its ability for rapid colonization of the environment. Moreover, it is interesting to note that induction of *CCD7* expression in low phosphate growth conditions is a conserved feature also reported in *P. patens* (Decker et al., 2017). Thus, we could imagine that SL in this species inhibits growth of the *protonema* and hastens switching to the reproductive stage when the medium contains suboptimal amounts of inorganic phosphate, thereby adapting growth to the soil phosphate content.

For the moment, we can only hypothesize that the same reflection can be applied to other extant mosses (except *Takakia*), as experimental evidence is lacking.

VIII-D) About the ancestral role(s) of SL

To date, there is not clear element refuting an ancestral role of SL as interspecific rhizosphere signals, although no consensus has been reached. These molecules would have been recruited as phytohormones later in the ancestry of seed plants, possibly under a new selection pressure faced by these plants. Indeed, players of the canonical SL signaling pathway seem to have been acquired all together either in seed plants or later in the Angiosperms lineage (this cannot be resolved yet owing to the inexistent data on SL response in Gymnosperms). The nature of this new selection pressure might be linked to the increasing complexity of branching forms/meristems. Moreover, outside of bryopsids mosses, most bryophytes are able to accommodate fungal symbionts, including the divergent moss *Takakia*, suggesting this ability is indeed ancestral and was lost in the moss lineage after the divergence of *Takakia* (Wang and Qiu, 2006). Given this unique role in symbiosis, there was no need for a SL signaling pathway in ancestral land plants.

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ANNEX

Annex 1 - Methods for medium-scale study of the biological effects of strigolactone-like molecules on the moss *Physcomitrella patens*

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This annex is a method chapter that is to be published in the Methods in Molecular Biology book series. It details some of the experiments used to generate results presented in chapters IV, VI and VII.

Running head: Methods for strigolactone studies in P. patens

i. Summary/Abstract

As a bryophyte and model plant, the moss *Physcomitrella patens (P. patens)* is particularly well adapted to hormone evolution studies. Gene targeting through homologous recombination or CRISPR-Cas9 system, genome sequencing and numerous transcriptomic datasets, have allowed molecular genetics studies and progresses in Evo-Devo knowledge. As to strigolactones, like for other hormones, both phenotypical and transcriptional responses can be studied, in both WT and mutant plants. However, as in any plant species, medium to large-scale phenotype characterization is necessary, owing to the general high phenotypic variability. Therefore, many biological replicates are required. This may translate to large amount of the investigated compounds, particularly expensive (or difficult to synthesize) in the case of strigolactones. These issues prompted us to improve existing methods to limit the use of scarce/expensive compounds, as well as to simplify subsequent measures/sampling of *P. patens*. We hence scaled up well-tried experiments, in order to increment the number of tested genotypes in one given experiment.

In this chapter, we will describe 3 methods we set up to study the response to strigolactones and related compounds in *P. patens*.

ii. Key Words

Economical, Phenotyping, Physcomitrella patens, Scale up, Semi-automated Strigolactones, Transcriptional response

1. Introduction

In this chapter, we present a scaled-up and economical twist for two widely used methods for characterizing hormones' effects on the moss *Physcomitrella patens* (*P. patens*). We also introduce a new experiment that can demonstrate even slight phenotypic response to hormones. The first method relies on vertical growth of the moss in the dark, which triggers specifically the elongation of caulonemal filaments upwards (negative gravitropism, as we previously described (*1*). Also see (2) for a recent review). Growth in the dark also enables to get rid of the potential interference of light with the response to strigolatone (SL)-like molecules of interest (*1*). We previously showed (*1*, *3*) that both natural SLs as well as widely used SL synthetic analogues enantiomeric mixtures such as *rac*-GR24 repress caulonema growth in a dose-dependent way in such experimental set-ups. The second method aims at testing the transcriptional response of *P. patens* to SL-like molecules of interest. It involves growth and subsequent incubation of *P. patens* tissues with the molecule(s)

of interest. RNA extraction is then carried out to eventually analyse the expression of selected SL-responsive genes by quantitative RT-PCR, with the adequate number of biological replicates. Finally, the third method is another means of testing phenotypic responses to SL-like molecules of interest by measuring phyllid regeneration ability (unpublished results).

In these three methods, the use of multi-well plates enables the observation of a response to minimal amounts of molecules, by concentrating plant treatment into small medium volumes.

2. Materials

All following media and tools must be sterile: use only plates/tubes from unopened sterile bags and ensure media and reusable tools have been autoclaved before starting experiments. Micro-elements and phosphate buffer stock solutions are stored in the fridge after being autoclaved (or alternatively filter-sterilized).

1. 1000x Micro-elements stock: 5.5 mg CuSO₄ 5H₂O, 5.5 mg ZnSO₄ 7 H₂O, 61.4 mg H₃BO₃, 38.9 mg MnCL₂ 4H₂O, 5.5 mg CoCl₂ 6H₂O, 2.8 mg KI, 2.5 mg Na₂MoO₄ 2H₂O, dissolved in 100 mL of MilliQ water. Store at 4°C.

2. 1000x Phosphate buffer stock: 25 g KH₂PO₄ dissolved in 100 mL of MilliQ water; pH7.0 adjusted with KOH

3. PpNH₄ solid medium (adapted from (4)): $0.8 \text{ g/L Ca(NO_3)4H_2O}$, $0.25 \text{ g/L MgSO_47H_2O}$, $12.5 \text{ mg/L FeSO_47H_2O}$,

1 mL/L microelements stock, 1 mL/L phosphate buffer stock, 0.5 g/L (NH₄)₂C₄H₄O₆, 7.2 g/L agar. Store at 4°C.

4. PpNO₃ solid medium (adapted from (4)): Ca(NO₃)4H₂O (0.8 g/L), 0.25 g/L MgSO₄7H₂O, 12.5 mg/L FeSO₄7H₂O, 1 mL/L microelements stock, 1 mL/L phosphate buffer stock, 10 g/L agar (*see* Note 1).

5. Solutions for treatments: For hydrophobic SL-like molecules, the primary solvent of choice is DMSO (*see* **Note 2**). The stock solutions of SL are hence prepared usually as 10 mM in 100% DMSO and can be kept at -20°C for long-term storage. Therefore, the control treatment consists of water-diluted DMSO at the same percentage as in SL treatments. These working dilutions of SL and DMSO are better prepared just before treatment but they can be stored at 4°C for several days (beware: freezing and thawing these "working solutions" is not advisable). A range of dilutions needs to be tested before the actual experiment, to demonstrate dose effect, and even more so when the SL-like molecule has never been tested on *P. patens* before. For instance, in *caulonema* growth experiments in the dark, the working range of (+)-GR24 spans from 0.01 μ M to 100 μ M, the most usually used concentrations being 0.1 μ M and 1 μ M.

6. Grinder and corresponding tips, as well as appropriate containers (sterile tubes or small pots) for grinding. Other means of grinding/tissue fragmentation can be preferred (*see* **Note 3**).

7. Micropore tape (3M, MicroporeTM).

8. Tissue-culture plates with 96, 24 and 6 wells. For 24-well plates: 3-4 plates for 1 genotype and 3 treatments (24-32 biological replicates for each genotype and treatment); for 6-well plates: 3-4 plates for 1 genotype and 3 treatments (6-8 biological replicates for each genotype and treatment).

9. Cellophane disks of two sizes: For standard round Petri dishes, a diameter of ~90 mm is needed (e.g. AA Packaging limited). For 6-well plates, a diameter of ~30 mm is needed (actual sizes depend on models and suppliers of plates/dishes).

10. Culture chamber: Unless otherwise stated, culture conditions will always be as following: long days (16 hours of day at 25°C and 8 hours of night at 23°C), 70 μ E fluence, 50% humidity. Dark incubation: same conditions except that the cultures are kept away from light by being doubly sealed in dark containers.

11. Liquid nitrogen.

- 12. Eppendorf tubes: 2 mL volume, screw lid, V-shaped bottom, skirted.
- 13. Ceramic beads (e.g. 1.4 mm diameter from MP Biomedicals).
- 14. Aluminium foil.

15. Very fine pliers (such as the 4A.SA.0 reference from Ideal-Tek). In addition, curved pliers might be more convenient for specific tasks (namely phyllid sampling), for which we advise to use pliers such as 7.S.0 from Ideal-Tek.
16. Micro-cutter (such as PrimerEdge® microsurgical knifes from Oasis).

3. Methods

All experiments described here start in the same manner, by obtaining axenic young *protonema* culture of *P. patens* as a tissue stock. To this end, you will need to prepare sterile PpNH₄ solid medium plates, overlaid with a cellophane disk, at least one plate per genotype. Tissues can be regrown on these plates from fragmented stocks or from dissected tissues (*see* **Note 4**). Fragmented stocks are obtained from 7-day old tissues collected with a sterile spoon, suspended in 20 mL of sterile water and ground with a Polytron homogenizer for 15-25 seconds. Use part (1/10 volume) of this stock to plate on a fresh Petri dish with cellophane.

From these tissues, it will take 1-2 weeks to obtain enough *protonema* material. Unless otherwise stated, all steps of the experiments are carried out in axenic conditions (horizontal laminar flow hood and sterile solutions and tools). Refresh the stock regularly, preferably from spores (once a year). Finally, always try to carry out these experiments at the same time during the day, as the circadian cycle seems to have a major effect on plant responses to hormones. This cautionary statement is particularly true for regeneration experiments.

3.1 Testing P. patens phenotypic response to SL-like compounds: caulonema filaments growth in the dark

1. Prepare the 24-well plates: 3-4 plates per genotype and for three treatments (including the control one). Pour 2 mL of PpNO₃ medium in each well, so ~50 mL per plate. Let the medium polymerize under the hood with the lid off, as excessive condensation on the lid can increase risks of contaminations. Do not leave your plates unsealed under the flow for too long, otherwise the medium will quickly dehydrate. You will need at least 24 wells for each treatment, distributed across at least 3 different plates, to have proper biological replicates. For instance, if you would like to test one new SL-like molecule along with a negative control (diluted solvent) and a positive control (e.g. *rac*-GR24 or, even better, (+)-GR24), which makes up for three treatments, you will need a minimum of 3 plates per genotype (figure 1-1). Start cultures: In each well of the 24-well plates, deposit a small piece of *protonema* from your PpNH₄ stock plate, at one extremity of the well (which will be the bottom side of the well from step 5 on). Try to always put the same amount of tissues in each well and always place it on the same side of the well. Whenever you finish a plate, put the lid back on and seal it with Micropore tape to avoid dehydration of samples and medium. Transfer your plates to the culture chamber for ~2 weeks, until *caulonema* filaments start to protrude from plants' periphery (check under the binocular).

3. Prepare treatments: Under the hood, mix your stock solution of SL (or stock DMSO solution for the control treatment) with sterile MilliQ water to your chosen working concentration. If you cannot proceed to step 4) immediately, keep your working solutions in the fridge. You will need 100-200 µL per well for the treatment.

4. Start treatments: first, remove the Micropore from every plate and note on the lid where each treatment goes (figure 1-1). Apply a volume of $100-200 \,\mu$ L of treatment solution directly upon each individual. When a plate is filled,

re-seal it and carefully swirl it to spread the treatment across the well. Transfer your plates vertically in the dark container and put them in the culture chamber for ~ 10 days.



Figure 1-1 – Layout of 24-well plates

5. Imaging: after the dark incubation is finished, the plates are unsealed and immediately imaged using an axiozoom (Zeiss) with a dedicated program taking a single picture for each well. For convenience, images may be converted to RGB before analysis. Possible results are shown in figure 1-2.

6. Measuring: using ImageJ, filaments are enumerated and the length of the three longest filaments is measured, for each well. Choose the appropriate test, depending mainly on your number of replicates, and proceed with statistical analysis.



Figure 1-2 – Instance of dark-grown *caulonemata* and their response to SL-like molecules. Depending on the molecule tested and its concentration, a whole range of phenotypic response can be observed in this experimental setup. The instance provided here shows the *Ppccd8* SL-deficient mutant response to (+)-GR24 at 0.1 μ M (central picture) and

 $1 \ \mu M$ (picture on the right). You can note that this molecule decreases both the number and length of *caulonema* filaments, in a dose-dependent manner. Indicated percentages reflect the effect of the molecule on the number of *caulonema* filaments.

3.2 Testing P. patens transcriptional response to SL-like compounds

1. Prepare the 6-well plates: 3-4 plates per genotype and for three treatments (including the control one). You will need to pour 6 mL of PpNO₃ medium in each well. Let the medium polymerize under the hood with the plate's lid off. Please keep in mind that you will need at least 6 wells for each treatment, distributed across at least 3 different plates, to have proper biological replicates. So, in the instance where you would like to test one new SL-like molecule along with a negative control (diluted solvent) and a positive control ((+)-GR24), which makes up for three treatments, you will need a minimum of 3 plates per genotype (figure 1-3).



Figure 1-3 – Layout of 6-well plates

2. Prepare the cellophane disks for your 6-well plates. You need a cellophane sheet and a tool to cut it in the right format. We typically use a scrapbooking punch producing disks with a diameter of 30 mm. Place the cellophane disks in a heat-resistant closed container and spread them to prevent stacking of the cellophane disks. Autoclave.

3. Ensure the medium is completely polymerized by slightly shaking the last plate you poured. After checking that, you can place the cellophane disks in the wells: put sterile MilliQ water in the sterile container with your cellophane disks. Using sterile pliers take one cellophane disk at a time from the water and try to lay it flat on the medium in the well (without trapping air bubbles underneath). Store your closed multi-well plates under the hood and proceed to step 4 as soon as possible.

4. Start cultures: In each well of the 6-well plates, deposit ~ $500 \,\mu$ L of freshly ground tissues. Whenever you finish a plate, put the lid back on, seal it with Micropore tape and carefully swirl the plate to evenly distribute the tissues in the wells.

5. Incubate the plates in the culture chamber for ~ 2 weeks. Check from time to time that there are no contaminations under the binocular.

6. Transfer the plates in the dark and let the tissues grow for at least one more week under the same temperature and hygrometry conditions (*see* **Note 5**).

7. Prepare treatments: Under the hood, mix your stock solution of SL (or stock DMSO solution for the control treatment) with sterile MilliQ water to your chosen working concentration. If you cannot proceed to step 8 immediately, keep your working solutions in the fridge. You will need 500 µL-1 mL per well for the treatment.

8. Start treatments: you must stay in "dark" conditions, so we usually treat tissues under the hood with green light only. First, remove the Micropore tape from every plate and note on the lid where each treatment goes. Follow the same overlay for each plate. Put the plates in the order of your choice (a logical order that is easy to remember and that you do not need to write down). When you are ready to start, note down the starting time. After you have finished the treatment, note down the ending time. Put your plates back in the dark container and in the culture chamber for 6 hours (*see* **Note 6**).

9. Prepare for tissue sampling: have as many screw Eppendorf 2 mL sterile RNAse-free tubes ready as you have wells, label them and place one clean ceramic bead per tube. Keep the tubes closed until sampling (*see* **Note 7**).

10. Sample tissues: ensure you have enough liquid nitrogen at your disposal before starting. You must also carry out the sampling under green light, but sterile conditions are no longer mandatory. Note down the starting time and the ending time for the sampling: duration of sampling should be roughly equal to the duration of the treatment delivery step, to ensure tissues were in contact with the treatment for the same time. Ensure you sample in the same order as you treated. Using a clean small spoon, gather the tissues from each well, quickly put them in the corresponding tube and immediately transfer the tube to liquid nitrogen. After the total \sim 3-week-growth of *P*. patens, you usually harvest a mix of several different tissues: *protonema*, gametophores and rhizoids. The RNA extracts you subsequently obtain will thus not be tissue-specific. When you have finished harvesting, transfer frozen tissues in a -80°C freezer (*see* **Note 8**).

11. Proceed to RNA extraction, cDNA preparation and quantitative PCR, following proper guidelines (such as the ones specified by Exner (*5*)). In order to select the appropriate reference genes for your qPCR experiments, you may follow advice from Le Bail *et al.* (*6*). We routinely use *PpAPT* (Pp3c8_16590) and *PpACT3* (Pp3c10_17080) as reference genes.

3.3 Testing P. patens phenotypic response to SL-like compounds: regeneration of dissected phyllids

This protocol is adapted from a previous protocol developed by Yoshikatsu Sato from NIBB (see related page "Regeneration of *protonemata* from excised leaves" on the PHYSCObase website: <u>http://moss.nibb.ac.jp/protocol.html</u>), previously used by Li *et al (7)*.

1. Prepare your *P. patens* tissues: for each genotype, you need at least one new $PpNH_4$ plate. From a $PpNH_4$ stock plate of each genotype, dissect 20-30 *protonema* pieces and transfer them to the new $PpNH_4$ plate. Ensure all stocks are approximately the same age and not too old (3-week-old as the maximum). Seal the plates and let them grow for at least 2 weeks, until you can see several gametophores per individual (e.g. per original piece of *protonema*).

2. Prepare your 96-well plates: You need 24 wells per genotype and treatment and thus 1 plate per genotype for three to four treatments (including the control one). You can multiply the number of plates if you wish to measure regeneration at several time points. We usually assess regeneration after 48h and 72h, but the 72h and 96h time points can be favoured depending on the molecule tested. For instance, we have shown that (+)-GR24 inhibits regeneration in a dose-dependent manner (unpublished results), thus later measurements can be more informative in this case. You need to pour 200 μ L of PpNH₄ medium in each well, so ~20 mL per plate. Let the medium polymerize under the hood with the plate's lid off, as excessive condensation on the lid can increase risks of contaminations. You will need at least 24
wells for each genotype and treatment. So, in one given plate (corresponding to one genotype), you are able to test up to 4 treatments: a negative control (diluted solvent), a positive control ((+)-GR24) and two SL-like molecules. Do not prepare these plates too much in advance, as the medium dries out very quickly in such small wells (*see* Note 9).

3. Prepare treatments: under the hood, mix your stock solution of SL (or pure DMSO for the control treatment) with sterile MilliQ water to your chosen working concentration. If you cannot proceed to step 4 immediately, keep your working

solutions in the fridge. Remember that you will need 50 μ L per well for the treatment and that this assay is more sensitive than *caulonema* growth (for (+)-GR24, effects have been observed starting at the minute concentration of 3 nM).

4. Distribute the treatments in the wells (figure 1-4). You have to do so before starting *P. patens* dissection, for two reasons: firstly, the overlay of liquid treatment will ease tissue deposition into the wells. Secondly, if you treat the tissues after deposition you risk introducing further variability in treatment duration (from the time of dissection) between your different samples.



Figure 1-4 – Layout of 96-well plates

5. Deposition of *P. patens* phyllids into the wells (figure 1-5): before all, gametophores of a given genotype are carefully collected and put aside in sterile MilliQ water. Use gametophores that are approximately the same age (for instance, only the ones growing from the centre of each plant). Note the time when you begin cutting, as well as the order you choose amongst genotypes and treatments. Then, using a micro-cutter, cut phyllids transversally near their connection point with the gametophore's stem. Immediately after cutting, carefully transfer each phyllid to the liquid treatment in a well by scooping it from below with pliers. Try to use phyllids that are approximately the same age, e.g. that grow at the same height on gametophores. Do not use wounded phyllids as they will display ectopic regeneration and thus must not be used in analysis (*see* **Note 10**).



Figure 1-5 – Global scheme of phyllid excision procedure

6. Incubate your plates in dark containers in the culture chamber, separating plates to be harvested at different time points into independent containers.

7. Observation of phyllid regeneration at 48h: phyllids are observed under a binocular at 48h after the beginning of the cutting step, in the same order as deposition. Regeneration is highlighted by the phyllid cell change in identity (de-differentiation) to that of a *chloronema* "stem" cell to give rise to a protruding *chloronema* filament (figure 1-6). Regeneration is assessed by two measurements: the percentage of regenerating phyllids and the number of regenerating filaments per phyllid (*see* **Note 11**).

8. Observation of phyllid regeneration at 72h: likewise, the percentage of regenerating phyllids and the number of regenerating filaments per phyllid are scored. Additionally, the number of cells per regenerated filament can be surveyed, as it can help highlight differences between samples (a divergence in regeneration ability can stem from a difference in cell division speed for instance). Choose the appropriate tests, depending mainly on your number of replicates, and proceed with statistical analysis.



Figure 1-6 – **Kinetics of phyllid regeneration.** Regeneration of *chloronema* filaments from the cells bordering the cut is assessed every 24 hours. Localized regeneration is usually a slow process and the first filaments are most often seen only from the 48h/72h time points on. This figure shows two instances of WT phyllid regeneration in the same culture conditions (with no specific treatment): panel A displays the usual regeneration process when the phyllid is cleanly cut. Panel B displays the ectopic regeneration of a phyllid that was wounded during handling. The black star underlines ectopic hollowing of cells that precedes regeneration of a filament. In both panels, regeneration *loci* are pointed by black arrows.

4. Notes

1. The agar must have high water-retaining ability, especially for method 3.1 where mosses are grown vertically, also its salt composition should be adapted to plant culture (for instance, we use 10 g/L Phytoblend agar from Caisson labs).

2. Acetone can also be used but tends to evaporate and thus solutions must be used immediately in this case.

3. Light grinding with a mortar and pestle in a small volume of MilliQ sterile water can also be employed. Fragmented tissues can then be transferred to a sterile tube or pot.

4. If you re-start your cultures from spores (advised after numerous cycles of fragmentation, *e.g.* once a year) it will take much longer to obtain *protonema* tissues at the proper stage. Usually, spores take 1-2 weeks to germinate and need at least 1 additional week to give rise to a sufficient amount of *protonema*. Then, the *protonema* needs at least one cycle of grinding followed by a 1 week-long culture before you can use it in your experiment).

5. You may transfer your plate in the dark while placing it upside-down. This can help further limit the risk of contamination.

6. Treatment duration may be adapted, though our previous experiments have shown that early SL response genes are the most differentially expressed after a 6 hour-long *rac*-GR24 treatment.

7. If you do not have a bead grinder and/or you do not have a lot of samples, you can instead prepare aluminium pockets for your samples.

8. If you do not harvest in tubes but in aluminium pockets, you can directly sample the tissues with the underlying cellophane disk using clean pliers, rather than use a spoon, and transfer the pocket to liquid nitrogen. Following this method of sampling, you can then finely grind the frozen samples in liquid nitrogen using a mortar and pestle.

9. If you have only three treatments to test, it is advisable to use 4 columns, *e.g.* 32 wells, for each treatment.

10. You can also put more than one single phyllid in each well but, while it will strengthen the statistical value of your results, it might slow down observations of the regeneration process.

11. If occasional ectopic regeneration occurs despite your extreme carefulness at the sampling stage, it is best not to record it and to only focus on regeneration at the cut. If you have enough replicates, you can also choose to completely overlook wounded leaves.

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Annex 2 – Detailed explanation of CRISPR-Cas9 use in this thesis work.

In all cases where the CRISPR-Cas9 tool was used in the present thesis work, transformation was carried out on *P. patens* protoplasts obtained from young *protonema* (5-7 day-old), so as to limit the risk of mutating diploid/polyploid cells, as endoreduplication is more prevalent in older filaments (Lang et al., 2018). The classical PEG/ heat-shock mediated protoplast transformation method was used, with minor modifications. Transformed protoplasts were left to regenerate their cell walls overnight in the dark, then cultivated for 5-7 days on non-selective solid medium with controlled osmolarity (8.5% mannitol), in an alginate mixture polymerized by adding CaCl₂ to ensure proper mechanical support for regeneration of protoplasts, in control long days conditions. When regeneration is ongoing, the alginate layer containing the regenerants is transferred on classical PpNH₄ medium containing the geneticin antibiotic (G418, 50 μ M) for one week. The regenerants that survived this selection step are then cultivated on non-selective PpNH₄ medium and genotyped. In our experiments, we used an independent plasmid containing the selection gene for resistance to G418, along with the plasmid permitting expression of a nuclear-targeted (SV40 NLS), codon-optimized version of Cas9 from *Streptococcus pyogenes* (Lopez-Obando et al., 2016b). Other plasmids targeting specific loci in *P. patens* genome were added, depending on the aim of the transformation.

2-A) Use of the CRISPR-Cas9 system for mutagenesis:

The first strategy we employed to generate mutations in the *PpSMXL* genes was a classical one using only one guide RNA. This guide was designed specifically for each gene, to target the uppermost possible locus in the CDS: at the beginning of the fourth exon for *PpSMXLA* and *PpSMXLB*, at the start of the second exon for *PpSMXLC* and only 80bp downstream of the START codon for *PpSMXLD* (see figure VII-1). Plasmids containing the resulting sgRNAs were used in combinations of one to four, to target from one to four loci at once (along with plasmids expressing Cas9 and the selection marker, Figure 2-1). This first strategy is analogous to the one employed to generate mutations in the *PpKAI2-L* genes (see chapter IV).

The second strategy was a test to replicate the effects of classical HR (homologous recombination) strategy without the need for removal of a selection cassette inserted in the genome in place of the knocked-out gene. This also prevents the risk of insertion of the cassette in several copies at the targeted locus (Cove, 2005). This use of the CRISPR system was devised following the advice from Fabien Nogué, a colleague at the IJPB and expert of the CRISPR system in moss. Moreover, it was later supported by publications from independent research teams working on this system in *P. patens*, concluding that targeting multiple sites within a single region can produce larger deletions (Mallett et al., 2019). For this purpose, guide RNAs were designed in the ~600bp of UTR closest to the START/STOP codon, for each *PpSMXL* gene. Plasmids containing the resulting sgRNAs were used in combinations of two to eight, to target from one to four loci at once (along with plasmids expressing Cas9 and the selection marker, Figure 2-1).

This second method did not work out for the generation of simple $Ppsmxl\Delta a$ and $Ppsmxl\Delta c$ mutants, nor for double $Ppsmxl\Delta a\Delta b$ mutants. Also, no triple or quadruple $Ppsmxl\Delta$ mutants were obtained. Hence, we used a single guide targeting ~150bp downstream of the START codon for PpSMXLA and 6 guides (the two targeting the UTRs + 4 new guides targeting the CDS) for PpSMXLC. Interestingly, the guide targeting PpSMXLC 5'UTR appeared to work

better in combination with other guides so we could eventually obtain $Ppsmxl\Delta c$ mutants. This guide also seemed more efficient when used in combination with guides against PpSMXLD, as we readily obtained $Ppsmxl\Delta c\Delta d$ double mutants.

Similarly, to what was done for *PpSMXLC*, the *PpMAX2* locus was mutated using 6 guide RNAs used simultaneously, albeit none was directed against the 3'UTR region (one guide in the 5'UTR just upstream of the START codon, 4 in the first half of the CDS and one in the second half, see Supplemental Figure VI-21).



Figure 2-1 – **Scheme of CRISPR-Cas9 mediated mutagenesis on** *Physcomitrium patens* **protoplasts.** GOI stands for gene of interest, G418^R for resistance to G418 (geneticin), gRNA for guide RNA, proAct for rice actin 1 promoter.



Figure 2-2 – **Identification of** *Ppsmxl* **mutants.** Mutations resulting from these two mutagenesis strategies were genotyped as follows: (A) and (B) *Ppsmxl*: A single pair of primers was used to amplify ~500bp around the site targeted by (the) guide RNA(s) (PCR gRNA). This fragment was then sequenced to identify mutations. Occasionally plants generating WT and mutated fragments (potential aneuploids) were identified. (C) and (D) *Ppsmxl*: Two PCR were carried out to check for the loss of the *PpSMXL* locus. Firstly, primers recognizing the 5'UTR and 3'UTR were used together (PCR UTR): in WT, the resulting amplicon was often too long to be obtained; in regenerants having lost the *PpSMXL* gene, the amplicon was ~800bp and was sequenced to confirm junction of the two UTRs. A second PCR was employed to amplify a ~450bp fragment of the CDS. This fragment should not be present in knock-out lines, therefore lines generating both types of amplicons were treated as potential aneuploids.

Chimeric nature of some transformants was also noted using the first mutagenesis strategy. However, it did not prove to be an issue as enough mutants were obtained (at least 2 mutants for each desired combination: *ab, cd*). In the case of the second strategy, the issue was somehow more prevalent, either because of chimerism (mix of WT and mutated cells) or aneuploidy. To isolate mutated cells, these regenerants were submitted to another round of protoplastization. However, no "pure" *Ppsmxl\Delta a \Delta b, Ppsmxl\Delta a* and *Ppsmxl\Delta d* mutants could be recovered after this "isolation" step (WT copies of the *PpSMXL* genes were still present thus they were probably aneuploids). Moreover, in the case of original *Ppsmxl\Delta a \Delta b* lines, recombination between the *PpSMXLA* and *PpSMXLB* loci was sometimes observed, undoubtedly linked to their very similar DNA sequences (homeology).

2-B) Use of the CRISPR-Cas9 system for knock-in facilitation:

As the HR process is initiated by double strand breaks in the DNA, we reasoned that facilitation of these breaks at a specific locus in *P. patens* genome, using the CRISPR system, could ease insertion of a construct at this locus by HR (knock in) (Figure 2-3). Hence, guide RNA targeting the Pp108 homology regions, outside of the sequence contained

in the insert, were employed along with the previously described Cas9 and selection plasmids and with a plasmid containing the desired construct (Figure 2-1, case 2B). This method was employed to generate transgenic lines described in chapter VI (*proZmUbi:GFP-PpSMXL, proZmUbi:flag-GFP* and *proPpSMXL:GUS*), as well as *proZmUbi:PpSMXL-GFP*, *proZmUbi:GFP-PpSMXLmut* and *proZmUbi:PpSMXLmut-GFP* lines (expressing PpSMXL proteins where the degron/degron-like motif is replace by LVGI).

Theoretically, the use of the selection plasmid carrying the geneticin resistance gene was not necessary, as the pMP vectors contain a kanamycin section marker, inserted at the Pp108 locus along with the transgenic construct. However, geneticin selection often gives more clean-cut results, perhaps because WT *P. patens* Gransden laboratory strains readily develop Kanamycin resistance themselves. Hence, we kept the same method of transformants selection as used for mutagenesis.



Figure 2-3 – Scheme of CRISPR-assisted insertion of transgenic construct in the genome of *Physcomitrium patens* (Knock-in)

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Titre : Perception et transduction du signal strigolactones chez la mousse Physcomitrium patens

Mots clés : Strigolactones, phytohormone, bryophytes, mousse, signalisation, SMXL

Résumé : Les strigolactones (SL) sont une nouvelle classe de phytohormones, retrouvées chez toutes les plantes terrestres. Chez les plantes vasculaires, les SL ont un rôle hormonal prédominant dans la régulation de l'architecture aérienne, mais aussi une fonction de signal symbiotique, favorisant ainsi la captation d'eau et de nutriments du sol par les plantes. Ces deux fonctions ont conduit à l'hypothèse selon laquelle les SL ont pu être essentielles dans les processus de colonisation du milieu terrestre par les plantes il y a 450 millions d'années. L'étude de la biosynthèse et de la signalisation des SL chez la bryophyte Physcomitrium patens (P. patens, non-vasculaire), par comparaison avec ce qui est connu chez les plantes vasculaires, permet de questionner l'évolution des voies cellulaires associées aux SL chez les plantes terrestres. Chez les plantes vasculaires, les voies de biosynthèse et de perception des SL sont assez bien décrites. La voie de signalisation des SL commence par la perception de la molécule par le récepteur D14 dans le cytosol, qui la clive et reste associé à une partie de la SL. Ce complexe interagit dans le noyau avec deux partenaires : la protéine à boîte F MAX2, capable de recruter un complexe d'ubiquitination, et certaines protéines SMXL. Ces protéines agissent comme des répresseurs de la réponse aux SL et vont être ubiquitinées sous l'action du complexe recruté par MAX2, puis rapidement dégradées par le protéasome. Chez P. patens, la plupart des gènes de biosynthèse et de signalisation des SL sont retrouvés, parfois en nombres différents comparés aux plantes vasculaires. Seules des approches de génétique inverse permettent de définir précisément leur fonction. La précédente caractérisation du mutant de biosynthèse Ppccd8 chez P. patens a montré que les SL sont synthétisées via une voie similaire à celle des plantes vasculaires. En outre, la fonction des SL dans la régulation de l'architecture est conservée chez P. patens. Au contraire, en accord avec la découverte précédente que l'unique homologue de MAX2 chez

P. patens n'est pas impliqué dans la signalisation des SL, les résultats ici présentés indiquent que la signalisation des SL n'est que partiellement conservée entre *P. patens* et les plantes vasculaires. En effet, seuls 2 homologues *PpKAI2L* de *D14*, sur les 13 possédés par *P. patens*, sont impliqués dans la perception des SL, d'après l'étude de mutants perte-de-fonction et les analyses de biochimie. Par protéines ailleurs, les PpKAI2L sont phylogénétiquement plus proches d'une autre protéine appelée KAI2 que de D14. Or KAI2 ne perçoit pas les SL chez les plantes vasculaires. De plus, la caractérisation des mutants perte-defonction *Ppsmxl*, obtenus par l'utilisation de la technologie CRISPR, et les analyses de liaison génétique avec PpMAX2 et PpCCD8 montrent que les quatre protéines PpSMXL ne jouent pas un rôle majeur dans la réponse aux SL. Cependant, les protéines PpSMXL les plus proches SMXL phylogénétiquement des ancestrales apparaissent comme des régulateurs importants de la croissance, ce qui pourrait constituer le rôle ancestral des protéines SMXL, en accord avec des études phylogénétiques récentes. Cette régulation de la croissance constituerait la réponse à un autre signal endogène, plus ancestral que les SL, le KL (ligand de KAI2). La transduction du signal KL serait conservée au moins chez les plantes terrestres et impliquerait chez P. patens certaines protéines PpKAI2L et les protéines PpMAX2 et PpSMXL. L'identité moléculaire du KL n'a pas encore été élucidée. N'étant pas conservée chez P. patens, la signalisation des SL résulte possiblement d'une innovation spécifique de la lignée des plantes vasculaires. Ainsi, la voie de réponse aux SL présente chez la mousse résulterait d'une évolution convergente vers la perception des SL. Il reste donc à élucider comment le signal SL est transduit chez P. patens, en aval de sa perception par certaines protéines PpKAI2L.



Title: Strigolactones perception and signal transduction in the moss *Physcomitrium patens*

Keywords: Strigolactones, phytohormone, bryophytes, moss, signaling, SMXL

Abstract: Strigolactones (SL) make up a novel class of phytohormones that are found across the whole land plant lineage. In vascular plants, the main hormonal role of SL is the repression of shoot axillary branching. However, SL are also a major symbiotic signal, granting the plant increased access to the nutrients and water contained in the rhizosphere. These two functions of SL led to the hypothesis that these molecules have been instrumental at the time of land colonization by plants, approximately 450 million years ago. Studying SL biosynthesis and signaling in the bryophyte Physcomitrium patens (P. patens, a non-vascular plant), and comparing these processes with the available knowledge in vascular plants, enables to investigate the evolution of SL cellular pathways in land plants. As a matter of facts, SL biosynthesis and signaling pathways are quite extensively described in vascular plants. Notably, SL signaling starts in the cytosol where the SL molecule binds to the D14 receptor. D14 cleaves the SL and stays covalently linked to a part of the SL. Under this conformation, D14 can then interact with two partners in the nucleus: the MAX2 F-box protein and SMXL proteins. SMXL proteins act as repressors of the SL response, as their interaction with D14 and MAX2 will trigger their ubiquitination and subsequent proteasomal degradation. Most SL biosynthesis and signaling genes have homologs in P. patens genome, sometimes in different numbers. Nevertheless, only reverse genetics approaches can clearly establish these homologs function. Previous characterization of *P. patens* SL deficient mutant Ppccd8 revealed that SL biosynthesis is broadly conserved between mosses and vascular plants. Furthermore, SL play a similar role in the regulation of plant architecture in *P. patens* as demonstrated in vascular plants.

On the other hand, results presented herein show that SL signaling is only partly conserved between P. patens and vascular plants, supporting the prior observation that the sole MAX2 homolog in P. patens is not needed for SL response. Indeed, only 2 out of the 13 P. patens D14 homologs (PpKAI2L genes) are involved in SL perception according to the characterization of Ppkai2/ CRISPR knock-out mutants and to biochemistry analyses. Moreover, instead of D14, PpKAI2L proteins are closer to the KAI2 protein, which is not involved in SL perception in vascular plants. In addition, the phenotype of CRISPR knock-out mutants for the *PpSMXL* genes, together with genetic linkage analysis of *PpSMXL* with *PpMAX2* and *PpCCD8*, show that none of the 4 PpSMXL proteins play a major role in SL response. However, the two PpSMXL homologs that are closer to the ancestral land plants SMXL seem to be important regulators of growth, which as per recent phylogenetic studies could be the ancestral role of the SMXL family. This effect on growth would actually be the main response to the ancestral KL (KAI2-ligand) signal, an endogenous signal different from SL. Transduction of the KL signal would hence be conserved across land plants and would be achieved in *P. patens* via some PpKAI2L proteins, together with the PpMAX2 and at least two PpSMXL proteins. To date, the identity of the KL molecule(s) remains under debate. As SL signaling is not conserved in *P. patens*, it appears that the known SL signaling pathway results from a vascular plants specific innovation. Likewise, SL response in *P. patens* would be the product of a convergent evolution. Therefore, the question as to how P. patens transduces the SL signal, downstream of perception by specific PpKAI2L proteins, remains open.