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The plant immune system: induction, memory and de-priming of defense responses by endogenous, exogenous and synthetic elicitors

Kay Gully

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Par

Kay Christian Gully

The plant immune system -
induction, memory and de-priming of defense responses by endogenous,
exogenous and synthetic elicitors.

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Rapporteurs avant soutenance :

Brigitte Mauch-Mani Professor, University of Neuchatel
Thorsten Nürnberger Professor, University of Tübingen

Composition du Jury :

Président	David Macherel	Professor, University of Angers (FR)
Examineurs :	Brigitte Mauch-Mani	Professor, University of Neuchatel (CH)
	Thorsten Nürnberger	Professor, University of Tübingen (DE)
	Etienne Bucher	Directeur de recherche, INRA Angers (FR)
Dir. de thèse :	Etienne Bucher	Directeur de recherche, INRA Angers (FR)
Co-dir. de thèse :	Marie-Noelle Brisset	Directrice de recherches, INRA Angers (FR)

Invité(s)

Alexandre Degrave Maître de conference, AgroCampus Ouest Angers (FR)

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1. Abbreviations

aa	Amino acid
ABA	Absciscic acid
ASM	Acibenzolar-S-methyl ester (commercialized as Bion) (see also BTH)
BA	benzyl adenine, a cytokinin for growth media
BABA	Beta-aminobutyric acid
BAK1	BRI1 associated kinase 1
BIK1	BOTRYTIS-INDUCED KINASE1
BTH	Benzothiadiazole (commercialized as Bion)
bZIP	Basic leucin zipper transcription factors
CD2-1	C-terminal epitope of flagellin
cDNA	Complementing DNA
CDPK	Calcium-dependent protein kinase
CEP1	C-TERMINAL ENCODED PEPTIDE 1
CERK1	Chitin elicitor receptor kinase 1
cfu	Colony forming units
CLE	CLAVATA3/EMBRYO-SURROUNDING REGION
COI1	Coronatine-insensitive 1
Col-0	<i>Columbia-0</i> Ecotype
CRP	Cysteine-Rich Peptide
dag	Days after germination
DAMPs	Damage/danger associated molecular pattern
dap	Days after propagation
DDM1	DECREASE IN DNA METHYLATION 1
DET	Differently expressed transcripts
DORN1	DOES NOT RESPOND TO NUCLEOTIDES 1
DRM	DOMAINS REARRANGED METHYLTRANSFERASE
dsRNA	Double stranded RNA
eATP	Extracellular ATP
eDNA	Extracellular DNA
EFR	Elongation factor Tu receptor

EF-Tu	Elongation factor thermo unstable
EGF	Epidermal growth factor
elf18/26	18/26 amino acids peptide of the N-terminus of EF-Tu
ETI	Effector triggered immunity
ETS	Effector-triggered susceptibility
FER	Feronia , RALF receptor
FLC	FLOWERING LOCUS C
flg22	22 amino acids peptide of the N-terminus of flagellin
FLS2	Flagellin sensing 2
FRK1	Flagellin responsive kinase 1
GLV	Green-leaf volatiles
gRNA	Guide RNA
H3ac	Histone 3 acetylation
H3K4me2	Histone 4 lysine 4 di-methylation
H3K4me3	Histone 4 lysine 4 tri-methylation
H3K9ac	Histone 3 lysin 9 acetylation
H4ac	Histone 4 acetylation
HAC1	Histone acetyltransferase 1
HR	Hypersensitive response
HypSys	Hydroxyproline-containing glycopeptides
IBA	Indole-3-butyric acid, auxin used for growth media
INA	2,6-dichloro-isonicotinic acid
ISR	Induced systemic resistance
JA	Jasmonic acid
JA-Ile	Jasmonoyl-Isoleucine
LPS	Lipopolysaccharides
LRR	Leucin rich repeat
LysM	Lysine motif
MAMP	Microbe associated molecular pattern
MAPK	Mitogen-Activated Protein Kinase
MET1	METHYLTRANSFERASE1
miRNA	MicroRNA
MOM1	MORPHEUS´MOLECULE 1

mRNA	Messenger RNA
MS	Murashige & Skoog Medium
MTI	Microbe triggered immunity
NADPH	Nicotinamidadenine Dinucleotidephosphate
NPR1	NON-EXPRESSOR OF PR1
NRPD (Pol IV)	RNA polymerase IV
NRPE (Pol V)	RNA polymerase V
OD	Optical density
OGs	Oligogalacturonides
PAMPs	Pathogen associated molecular pattern
PCR	Polymerase chain reaction
Pep	Danger peptide
PEPR	Pep receptor
PGN	Peptidoglycans
PIP	PAMP-induced peptides
POL II	Polymerase II
PR	Pathogenesis-Related
PROPEP	Precursor of Pep
PROSCOOP	Precursor of SCOOP
PRR	Pattern recognition receptor
PSK	Phytosulfokines
<i>Pst</i>	<i>Pseudomonas syringae</i> pathovar <i>tomato</i> DC3000
PSY1	PLANT PEPTIDE CONTAINING SULFATED TYROSINE
PTI	Pathogen triggered immunity
PTMP	Small post-translationally modified peptides
qPCR	Quantitative polymerase chain reaction
RALF	Rapid alkalization factor
RAM	Root apical meristem
RbohD	Respiratory-burst oxidase homologue
RdDM	RNA-directed DNA methylation
RDR6	RNA-DEPENDENT RNA POLYMERASE 6
RGF	Root meristem growth factor
RLK	Receptor like kinase

RLP	Receptor like protein
RLU	Relative light unit
ROS	Reactive oxygen species
SA	Salicylic acid
SAM	Shoot apical meristem
SAR	Systemic acquired resistance
SCOOP	Serine rich endogenous peptide
scSCOOP12	Scrambled serine rich endogenous peptide 12
SE	Standard error
siRNA	Small interfering RNA
ssRNA	Single stranded RNA
TMV	Tobacco Mosaic Virus
UV	Ultraviolet
WAK1	Wall associated kinase 1
Ws	<i>Wassilijewska</i> ecotype

2. Summary

Due to the sessile nature, plants cannot simply escape a stressful situation. However, plants developed a multilayered immune system to counteract certain stresses. The immune system can for instance be triggered by herbivore feeding and pathogen or virus infections. Highly specialized components of the immune system enable the plant to detect a dangerous situation. These danger signals can have exogenous or endogenous origins. Exogenous danger signals derived from microbes are called microbe associated molecular patterns (MAMPs). The best studied MAMP is flg22, a conserved motif of the bacterial flagella. On the other hand, endogenous danger signals are referred to as damage/danger associated molecular patterns (DAMPs). Actively processed and/or secreted peptides upon an infection that modulate immune response are referred as phytochemicals. Next to their activity as defense amplifiers, phytochemicals have been shown to regulate developmental processes. In addition to these classes of exogenous and endogenous elicitors there are also synthetic elicitor molecules. These molecules are known to induce a systemic defense activation at distant non-challenged sites. This effect is called systemic acquired resistance (SAR). Along with SAR comes an extensive transcriptional reprogramming of defense-related genes and gene priming. Priming describes a mechanism in which a subset of genes is kept at a “ready-state” to facilitate a subsequent transcriptional regulation. Priming is often connected to epigenetic regulation of gene expression.

In this work, I describe the discovery and characterization of a novel phytochemical. I will begin with the bioinformatics-based discovery of a peptide family that we termed SCOOP (for Serine rich endogenous Peptide). I then show that a peptide, covering a conserved motif present in all members of the SCOOP family, induces various defense responses in *Arabidopsis*. Moreover, I show that the SCOOP12 peptide promotes resistance against *Pseudomonas syringae* and I demonstrate that perception of the SCOOP12 relies on the BAK1 coreceptor. I show the SCOOP peptide family contains several members apart from SCOOP12 that induce defense responses. Finally, I present a role of the SCOOP peptide family in plant developmental processes. The knock-out mutant of the putative SCOOP12 precursor

proscop12 shows an increased root length while treatments with three different SCOOP peptides induce severe phenotypical changes in the root tissue.

In the second part I investigate the effect of two other classes of elicitors (exogenous and synthetic elicitors). I show that treatments with a synthetic elicitor (BTH, a salicylic acid analogue) can lead to long-term transcriptional memory at certain genes. I found that subsequent challenging of such plants with the exogenous elicitor flg22 reverted this transcriptional memory bringing their expression back to the original pre-treatment level. This memory behaviour we describe as “de-priming” memory response. I made very similar observations in apple (*Malus x domestica*), suggesting that this response is highly conserved in plants. Finally, I describe a potential role for DNA methylation in the observed transcriptional memory behaviour. I show that plants defective in DNA methylation pathways showed a different memory behaviour.

In conclusion, my thesis investigates effects on plant transcription, development and defense by endogenous (the SCOOP peptide family), exogenous (flg22) and synthetic (BTH) plant elicitors. My thesis shows (1) how diverse the function of these elicitors can be and (2) how the plant defense system can affect plant development and trigger memory.

2.1. Résumé en français

De par leur nature d'organismes sessiles, les plantes ne peuvent fuir une condition stressante. Toutefois, les plantes ont développé un système immunitaire multicouche pour contrer certains stress. Le système immunitaire peut, par exemple, être déclenché par le broutage d'herbivores ainsi que par des infections bactériennes ou virales. Des composés hautement spécialisés du système immunitaire permettent à la plante de détecter les situations dangereuses pour elle. Ces signaux de danger peuvent avoir des origines endogènes ou exogènes. Les signaux de danger exogènes sont dérivés des micro-organismes se nomment *microbe associated molecular patterns* (MAMPs). Le MAMP le plus étudié est flg22, un motif conservé présent dans les flagelles bactériens. D'un autre côté, les signaux de danger endogènes sont identifiés en tant que *damage/danger associated molecular patterns* (DAMPs). Les phytoctokynes sont, quant à elles, des peptides activement produits et sécrétés durant une infection et modulant la réponse. De manière conjointe à leur activité d'amplificateur des mécanismes de défense de la plante, les phytoctokines jouent un rôle dans le processus développemental. En plus de ces catégories d'éliciteurs exogènes et endogènes, il existe également des éliciteurs synthétiques. Ces molécules peuvent induire une activation des défenses systémiques sur des sites distants et non-infectés. Cet effet est dénommé résistance acquise systémique (SAR). Le SAR génère une reprogrammation transcriptionnelle extensive des gènes associés à la réponse défensive et au priming. Le priming décrit un mécanisme par lequel un petit nombre de gènes est maintenu dans un « état de réponse » pour faciliter une régulation transcriptionnelle subséquente. Le priming est souvent connecté à la régulation épigénétique de l'expression génique.

Dans ce travail, je décris la découverte et caractérisation d'une nouvelle phytoctokine. Je commencerai par présenter l'identification bioinformatique d'une famille de peptides que nous avons baptisé SCOOP (*Serine riCh EndOgenOus Peptide*). Je montrerai ensuite qu'un peptide, présentant un motif conservé par tous les membres de la famille SCOOP, induit des réponses défensives variées chez *Arabidopsis*. De plus, je mets en évidence que le peptide SCOOP12 induit une résistance contre le pathogène *Pseudomonas syringae* et démontre que la perception de SCOOP12 nécessite le corécepteur BAK1. Je présente également que la famille de peptides SCOOP contient plusieurs membres, autres que SCOOP12, qui induisent

une réponse défensive. Finalement, j'expose un rôle de la famille SCOOP dans le processus développemental de la plante. Le mutant *knock-out* du précurseur putatif de SCOOP12, *proscoop12*, présente une augmentation de la longueur des racines alors que des traitements avec d'autres peptides SCOOP induisent des changements phénotypiques sévères au niveau des tissus racinaires.

Dans la seconde partie, j'investigue les effets de deux autres classes d'éliciteurs (exogènes et synthétiques). Je montre que les traitements avec un éliciteur synthétique (BTH, un analogue de l'acide salicylique) peuvent conduire à une mémoire transcriptionnelle de longue durée de certains gènes. J'ai observé que le challenge expérimental de ces plantes avec l'éliciteur exogène *flg22* désactive cette mémoire transcriptionnelle en ramenant le niveau d'expression de ces gènes à leur niveau initial pré-traitement. Nous avons identifié ce comportement comme un « désamorçage » (*de-priming*) de la réponse-mémoire. J'ai effectué des observations similaires sur le pommier (*Malus x domestica*), suggérant que cette réponse puisse être hautement conservée chez les plantes. Finalement, je décris le rôle potentiel de la méthylation de l'ADN dans le phénomène observé de mémoire transcriptionnelle. Je démontre que les plantes avec des voies métaboliques de la méthylation de l'ADN défectueuses présentent un comportement mémoire différent.

En conclusion, ma thèse investigue les effets éliciteurs endogènes (la famille de peptides SCOOP), exogènes (*flg22*) et synthétiques (BTH) sur la transcription, le développement et les défenses des plantes. Ma thèse met en lumière (1) la diversité des fonctions de ces éliciteurs et (2) de quelle manière les mécanismes de défense des plantes peuvent affecter le développement et activer une mémoire.

3. General Introduction

Why working on plants? What makes it so important to work on plants and why is this research so relevant but still often underrated in a cross-border context of science? These are questions I often asked myself during the last years. However, during my studies I discovered how multifaceted, fascinating and unexpected this part of science can be. Why working on plants? – not only the work is fascinating but also important. Plants are the main source of energy to terrestrial ecosystems. Plants are capable to convert sunlight, CO₂ and water into sugar. Carbohydrates are therefore available for other organisms. Especially at the IRHS in Angers we profit a lot from this in the form of delicious apple fruits.

Because of their sessile nature, plants cannot avoid danger by simply moving away. Therefore, plants need to be protected in a different manner compared to animals. In fact, plants are home to millions of potentially disease-causing pathogenic microbes that can have a negative influence on plant growth. Whereas beneficial microbes can have positive effects (Delmotte et al., 2009). Despite this apparent challenging condition plants were able to entrench in most environments. As a key to success, plants have developed a very reliable defense system. This plant immune system consists of several layers of constitutive and inducible responses to fight back the millions of microbes (Jones and Dangl, 2006b).

Next to the constitutive defense system, which consists of properties such as the waxy cuticular that covers the leaf surface, trichomes, thorns, secondary metabolites which harm invading pathogens, as well as lignified cell walls, defense responses can be induced (Thordal-Christensen, 2003). One key to activating the plant immunity is the sensing of danger.

These danger signals can originate from the infectious agent or from the plant itself. According to the latest classification of these immunogenic agents, plant host factors can be divided into two categories. The damage-associated molecular patterns (DAMPs) and peptides which are actively processed and/or secreted upon infection in order to modulate the immune response (phytochemicals) (Gust et al., 2017).

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Whereas peptides derive from infectious agents are referred to as Microbe- Associated Molecular Patterns (MAMPs) or as Pathogens- Associated Molecular Patterns (PAMPs) one of the best characterized molecule among them is flagellin (Boller and Felix, 2009).

The activation of defense responses at a MAMP recognition site is followed by systemic defense activation even at distant non-challenged sites. This mechanism is called systemic acquired resistance (SAR). Along with SAR comes an extensive transcriptional reprogramming of defense-related genes and gene priming. Priming describes a mechanism in which a subset of genes is kept at a “ready-state” to facilitate a subsequent transcriptional regulation. Epigenetic mechanisms, such as DNA methylation and histone modification and their influence on the chromatin reconfiguration, are shown to have influence on plant adaptation to different biotic stresses (Espinosa et al., 2016).

3.1. MAMPs and DAMPs – defense inducing molecular signatures

Plants have developed mechanisms to detect various forms of danger, including the attack by pathogens as well as tissue and cellular damage. Since plants lack an adaptive immune system they have strong need for rapid detection of all kinds of pathogens. Thus, the perception of defense-inducing molecular signatures like MAMPs and DAMPs is viable for the fast initiation of defense responses. Exogenous as well as endogenous elicitors will be described in the following chapter.

3.1.1. Microbe-associated Molecular Patterns (MAMPs)

MAMPs are highly conserved and crucial molecules, often they are found in a whole clade of microbes. Formally MAMPs were referred as PAMPs. However, these structures are not exclusively restricted to pathogens and the term MAMPs is more accurate (Boller and Felix, 2009). MAMPs have the ability to elicit defense responses upon their perception. Prominent examples for MAMPs are peptidoglycans. These are the building blocks of the bacterial cell wall. Another example are bacterial elongation factors and finally flagellin monomers, which are required for the movement of motile bacteria (Newman et al., 2013; Choi and Klessig, 2016). The class of MAMPs can be separated according to their origin. Fungal MAMPs can also

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be perceived by plants. One of best studied receptors is the chitin elicitor receptor kinase 1 (CERK1). This receptor plays an important role in chitin triggered immunity (Miya et al., 2007; Wan et al., 2008). Next to chitin, ergosterol, which is an important building block of the fungus itself, can serve as defense elicitors (Klempner et al., 2014). Moreover, recently it was shown that the linear 1,3- β -D-glucans, which is also present in the walls of fungi and oomycetes, are recognized by the plant (Mélida et al., 2018). Additional MAMPs can derive from viruses. Viruses are often transmitted through vector organisms like aphids or nematodes. These organisms wound the plant by sucking or feeding on it and thereby transmit the virus into the plant cells (Bragard et al., 2013). Typical viral molecules that can be observed by the plant are virus-specific coat proteins, movement proteins or replicases (Conti et al., 2017). However, in contrast to other MAMPs, virus derived MAMPs mostly induce virus-specific defense responses. This includes the activation of the RNA silencing machinery and located cell-death in order to restrict viral spread (Padmanabhan and Dinesh-Kumar, 2014). Finally, bacterial MAMPs include the bacterial flagellin. Flagellin is the hallmark of a peptide MAMP (Felix et al., 1999). As mentioned before, flagellin monomers are the building blocks of the bacterial flagellum (Taguchi et al., 2008). The N- and C- terminus of this protein are highly conserved. It was shown that only nanomolar concentrations of a conserved part of 22 amino acids (aa) from the N-terminus of *Pseudomonas syringae pv. tomato (Pst DC3000)* flagellin elicits defense responses (Felix et al., 1999; Smith et al., 2004). This small conserved peptide is referred to as flg22 and is perceived by most plant species via the leucine-rich repeat receptor like kinase (LRR-RLK) FLAGELLIN SENSING 2 (FLS2) (Gomez-Gomez et al., 1999; Bauer et al., 2001; Chinchilla et al., 2006). Plants defective in FLS2 are completely “blind” to flagellin and were found to be more susceptible to *Pst. DC3000* infections. Interestingly, the exposure of Arabidopsis plants to flg22 results in a protective effect. Pre-treatments with exogenously applied flg22 leads to enhanced resistance to *Pst. DC3000* (Zipfel et al., 2004). However, not all epitopes of flagellin are as universal as flg22, for instance the flgII-28 has only been found to be perceived by several solanaceous species. A C-terminal epitope of flagellin, called CD2-1 has only been found to be perceived by rice (Cai et al., 2011; Veluchamy et al., 2014; Katsuragi et al., 2015). Although flg22 recognition by FLS2 is very efficient some microbial species or strains have diverged their MAMPs to render them unrecognizable by the plant. It was shown that the flg22 peptide covering flagellin of *Erwinia amylovora* does not elicit a defense response in Arabidopsis or only in very high concentration. However, only two

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mutations of FLS2 can increase the recognition of *E. amylovora* flagellin peptide (Helft et al., 2016). This indicates that plants can readily evolve in order to adapt to novel or modified microbes. Interestingly, flg22 can be transported by the plant into distal tissues. This process is established by endocytosis of flg22 together with the FLS2 and results in long-distance transport of flg22 (Jelenska et al., 2017).

Another well-known MAMP is the ELONGATION FACTOR THERMO UNSTABLE (EF-Tu). This MAMP is essential for elongation during protein synthesis (Jeppesen et al., 2005). EF-Tu is a highly conserved 18 or 26 amino acids (aa) (elf18/26) and shows a 90% homology between hundreds of bacteria species. EF-Tu is perceived via the PRR EF-Tu RECEPTOR (EFR) (Kunze et al., 2004; Zipfel et al., 2006).

Next to these examples also non-proteinaceous MAMPs like peptidoglycans (PGN), β -glucans and lipopolysaccharides (LPS) have been identified and shown to elicit defense responses (Proietti et al., 2014; Zipfel, 2014; Gust et al., 2017).

3.1.2. Damage/Danger-associated Molecular Patterns (DAMPs)

Peptides that are produced by the plant itself and trigger a defense response by the same plant are generally referred as DAMPs. However, with the increasing knowledge of the action of DAMPs the classification is more and more diverse. The endogenous danger signals can be divided into primary, "classical" DAMPs, which are passively released upon plant tissue damage and secondary endogenous danger signals. This second class can again be divided into (1) secondary endogenous peptides which are processed and released upon herbivore or microbial infections and induced plant defense responses and (2) secondary endogenous danger signals which are linked to the regulation of plant growth and development. These endogenous peptides are also referred to as phyto cytokines. However, a large proportion of phyto cytokines play roles in plant development and growth as well as in plant defense (Gust et al., 2017)

Primary endogenous danger signals are passively released upon wounding. Wounding and herbivore attacks results in the release of intracellular and cell wall-associated molecules into the apoplastic space (Mithöfer and Boland, 2012; Ferrari et al., 2013; Choi and Klessig, 2016; Duran-Flores and Heil, 2016). Well-known primary DAMPs include the oligomeric fragments

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of plant cell-wall pectin, termed oligogalacturonides (OGs). OGs are produced by the polygalacturonase enzyme. This enzyme is secreted by fungi during plant infection (D'Ovidio et al., 2004). OGs can be sensed by the plant with the wall-associated (receptor) kinase 1 (WAK1) (Brutus et al., 2010). Other molecules that are considered as DAMPs are extracellular (e) nucleotides like eATP. In *Arabidopsis* eATP is perceived by the lectin receptor kinase DOES NOT RESPOND TO NUCLEOTIDES 1 (DORN1) (Choi et al., 2014). Interestingly, eATP has strong influence on jasmonate (JA) signaling (Tripathi et al., 2018). JA signaling is typically induced upon infection by necrotrophic pathogens and chewing herbivores (Reymond and Farmer, 1998). Upon herbivory, the green-leaf volatiles (GLV) are released from wounded leaves (Scala et al., 2013). GLV have direct antimicrobial effects and can thus serve as primers of systemic immunity upon local damage. It was found that also fructans in fructans-accumulating plants have actions as primary DAMPs (Versluys et al., 2016).

Additionally, to passively released danger signals plants produce secondary endogenous danger peptides. These molecules are shown to modulate immune responses to herbivory and microbial infections (Albert, 2013; Mott et al., 2014). Typically, the molecules are produced as larger precursor proteins and are processed by proteolytic cleavage and then secreted upon wounding, microbial infection or MAMP treatment (Yamaguchi and Huffaker, 2011). One of the first danger peptides was initially discovered in tomato. There, systemin was shown to be an 18aa long polypeptide that is processed from a 200aa prosystemin precursor upon wounding and herbivory (Pearce et al., 1991; Ryan and Pearce, 2003). For many years the processing mechanism was unknown, but just recently, phytaspases and aspartate-specific proteases, have been found to be implicated in systemin processing (Beloshistov et al., 2018).

Systemin was shown to induce various defense reactions including the induction of the expression of protease inhibitors which are crucial for defense reactions against herbivores (Zavala et al., 2004; Zhu-Salzman et al., 2008; Hartl et al., 2011). In several solanaceous plants a second class of systemins are produced. They are referred to as hydroxyproline-rich systemins (HypSys) (Pearce et al., 2001b; Pearce et al., 2009). In *Nicotiana tabacum* two HypSys (*NtHySysI/II*) have been identified and shown to induce defense responses in a similar way that Systemin does while not sharing any sequence homology to the prosystemin identified in tomato (Pearce et al., 2001b; Pearce, 2011).

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Another family of endogenous danger peptides are the Arabidopsis PAMP-induced peptides (PIP). The *AtPIP* family harbors 11 members in Arabidopsis of which two members (*AtPIP1* and *AtPIP2*) have been shown to trigger immune responses similar to that of flg22 (Hou et al., 2014). *AtPIP1* and *AtPIP2* are produced as preproteins (*PrePIP1* and *PrePIP2*) upon microbial infection as well as MAMP treatment. The preproteins harbor a signal domain that enables the secretion in a signal peptide-dependent manner. *AtPIPs* are perceived by the receptor LRR-RK RLK7 (Hou et al., 2014).

Another peptide family that has been shown to be widespread throughout the plant kingdom are the rapid alkalization factors (RALFs). They have been identified in flowering plants, lycophytes and mosses (Murphy and De Smet, 2014). All 39 RALF peptides, encoded by the Arabidopsis genome carry a N-terminal signal peptide (Sharma et al., 2016). RALFs were first identified by their ability to cause alkalization of tobacco cell suspensions (Pearce et al., 2001a). Not all RALF peptides are the result of the cleavage of propeptides, those that require processing are processed during secretion or in the apoplast by the Arabidopsis subtilase SITE-1-PROTEASE (*AtS1P*) (Pearce et al., 2001a; Stegmann et al., 2017). Next to wide abundance of different plant species in which RALFs can be found, the high number of biological processes in which RALFs are involved is remarkable. Individual RALFs play roles in combining biotic and abiotic stresses, induce the plant defense, play roles in fungal infection resistance and act with elf18 to induce plant immunity (Gupta et al., 2010; Atkinson et al., 2013; Stegmann et al., 2017). RALF members have also been shown to be involved in controlling the pollen tube and therefore to be important in the regulation of sexual reproduction (Stegmann and Zipfel, 2017). The perception of *AtRALF23* is established by the malectin-like receptor kinase FERONIA (FER) (Stegmann et al., 2017).

Another family that has been well-studied and originally identified in Arabidopsis is the plant elicitor peptide (Pep) family. This family harbors 8 members named *AtPep1-8* (Huffaker et al., 2006; Yamaguchi and Huffaker, 2011; Bartels and Boller, 2015) and has been identified in various plant species (Lori et al., 2015). The precursors of the eight *AtPep* peptides (*PROPEP1-8*) have a tissue-specific expression pattern and the expression of individual members is induced by wounding, MAMP treatment, microbial infection and by treatments with their own *AtPep* peptides (Huffaker et al., 2006; Albert, 2013; Mott et al., 2014; Bartels and Boller, 2015). The *Atpep* peptides are perceived by two leucine-rich repeat receptor kinases, named

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AtPEPR1 and *AtPEPR2* (Yamaguchi et al., 2006; Krol et al., 2010; Yamaguchi et al., 2010). *AtPeps* are involved in various defense responses against bacteria, fungus, oomycetes as well as in responses to insect attacks (Huffaker et al., 2006; Yamaguchi et al., 2010; Huffaker et al., 2013; Yasuda et al., 2017). However, it is still not known how PROPEP proteins are processed and secreted. It has been proposed that the peptides are processed (probably by metacaspase 4 (personal communication with Tim Hander)) and released only upon wounding (Bartels and Boller, 2015). An indication on how wide-spread this peptide family is, was provided by recent work on *Rosaceae* species. In 95 *Rosaceae* varieties up to 180 PROPEP 1-4 sequences could be identified. Homologues of PROPEPs could be found in different varieties of apple, pear, peach and strawberry (Ruiz et al., 2018b; Ruiz et al., 2018a).

3.2. Phytocytokines and small endogenous signaling peptides

Secondary endogenous danger signals which are involved in plant growth and development, but are not primarily involved in plant defense responses, could be referred to as phytocytokines (Gust et al., 2017). According to this classification prominent small peptides including CLAVATA3/EMBRYO-SURROUNDING REGION (CLE), C-TERMINAL ENCODED PEPTIDE 1 (CEP1), ROOT MERISTEM GROWTH FACTOR (RGF), phytosulfokines (PSK), *AtPeps*, *AtPIPs* and RALF and many more belong to this group. The full overview of all endogenous signaling peptides is shown in Figure 1. *AtPeps*, *AtPIPs* and RALF belong to phytocytokines because, next to their involvement in defense responses, they have also been shown to influence plant growth and development (Hou et al., 2014; Murphy and De Smet, 2014; Gully et al., 2015). Several of these endogenous signaling peptides have also been shown to be involved in development and functioning of nodulation (Kereszt et al., 2018). Phytocytokines belong to small endogenous signaling peptides, they are also referred to as plant peptide hormones in order to separate them from classical plant hormones such as auxin, cytokinin and ethylene, which have been assumed to be the main players in cell to cell communication and intercellular signaling processes (Davies, 2004).

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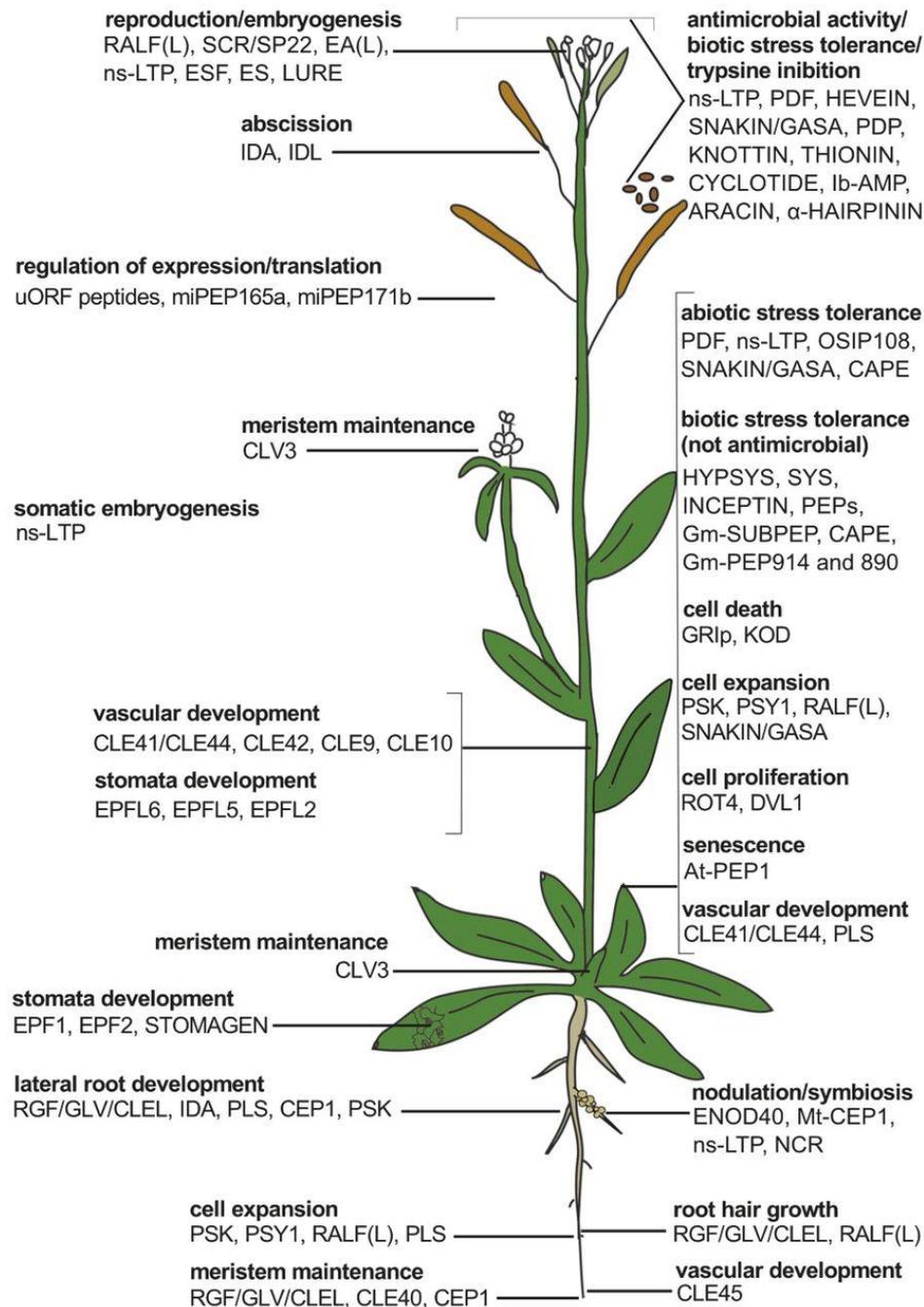


Figure 1: **Small endogenous signaling peptides mediates a high number of plant physiological responses.** This illustration of small endogenous peptides involved in several plant physiological processes was taken from (Tavormina et al., 2015). Not all small peptides presented in the figure are discussed in this introduction. For more information the original review as well as (Czyzewicz et al., 2013) provide an exhaustive overview.

In Arabidopsis, the CLE peptide (CLAVATA3/EMBRYO SURROUNDING REGION-RELATED) family is a large family with 32 members. *CLE* genes play various roles in stem cell homeostasis

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and in different types of meristems as well as many biological roles in growth and development (Wang et al., 2015; Dao and Fletcher, 2017). A common feature of all CLE members is that they all derive from a roughly 150aa long precursor protein containing an N-terminal secretion signal and the C-terminal 14aa long CLE motif (Cock and McCormick, 2001; Wang and Fiers, 2010). One of the best characterized CLE family members is CLV3 (Clavata 3). CLV3 is involved in the maintenance of the shoot apical meristem (SAM), while other CLE family members are involved in cell fate regulation in the root apical meristem (RAM) and (pro) cambium (Kondo et al., 2006; Betsuyaku et al., 2011; Matsubayashi, 2014; Czyzewicz et al., 2015). The perception of CLV3 is not yet been completely elucidated. Three major receptor kinase complexes have been proposed for signaling in the SAM, namely CLV1, CLV2, CORYNE as well as RPK2 (Clark et al., 1995; Kinoshita et al., 2010; Matsubayashi, 2014). However, in other tissues and for other CLE peptides the perception has been proposed to be established by other receptors (Betsuyaku et al., 2011). Interestingly, signaling of various CLE peptide family members have been shown to integrate with signaling of classic plant hormones (Wang et al., 2015). These interactions include auxin, brassinosteroids and cytokinin signaling (Whitford et al., 2008; Kondo et al., 2011; Kondo et al., 2014). The same observations have been made in other plant species apart from Arabidopsis. 47 CLE genes have been identified from rice (Kinoshita et al., 2007) one of which (*OscLE48*) has been shown to be induced by auxin application (Guo et al., 2015).

Many small signaling peptides are involved in root growth and root development. The number of identified peptides is steadily increasing for different root tissues (Oh et al., 2018). The C-TERMINAL ENCODED PEPTIDE (CEP) family was identified by a *in silico* approach and contains 15 members (Ohyama et al., 2008). One member, CEP1 is a 15aa long small peptide and has been shown to be involved in the regulation of lateral root growth by regulating root meristem activity (Ohyama et al., 2008). Moreover, CEP family members have been shown to lead to increased expression in response to Nitrogen (N) starvation. During N starvation CEPs are transported from the roots to the shoots and there perceived by two LRR-RLK receptors (CEPR1 and CEPR2). This leads to a systemic signal which results in the upregulation of N transporter genes in the roots (Tabata et al., 2014). The nature of this signal was recently uncovered, the two polypeptides CEPD1 and CEPD2 are transported from the shoot to the root (Ohkubo et al., 2017). Moreover, CEP members have been shown to be involved in the

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development of lateral roots (LR). *CEP1* overexpression leads to the inhibition of root growth and is therefore, in line with *CEP5*, a negative regulator of root growth (Ohyama et al., 2008; Roberts et al., 2016)

A peptide family mainly involved in the development of the primary root is ROOT MERISTEM GROWTH FACTOR (RGF). Members of this family play roles in the root meristem maintenance (Oh et al., 2018). RGF1 requires sulfation of a tyrosine residue to achieve its activity in the maintenance of root stem cells (Matsuzaki et al., 2010). The perception of RGF is not yet been investigated in great detail. The *rgf insensitive (rgi)* is a quintuple mutant consisting of the genes *PLETHORA1*, *PLETHORA2*, *RGFR1,2,3* (Ou et al., 2016; Shinohara et al., 2016). It is up to now not clarified whether all five receptors are equally involved in the perception of RGF peptides.

A well-known small peptide family are the phytosulfokines (PSK). PSKs were originally found to act as growth factors in low-density suspension cultures (Matsubayashi and Sakagami, 1997; Matsubayashi et al., 1999; Yang et al., 1999). In *Arabidopsis* five PSK precursor genes have been identified, coding for 80aa long precursor proteins (Yang et al., 2001). The PSK peptide is only 5aa long and was shown to regulate cell expansion in the root elongation and differentiation zone (Kutschmar et al., 2009). PSK has a close homologue, PLANT PEPTIDE CONTAINING SULFATED TYROSINE (PSY1) which is an 18aa long peptide and that has been shown to possess similar functions as PSK (Amano et al., 2007). PSK is recognized by two LRR-RK, PSKR1 and PSKR2 while PSY1 is perceived by the close homologue PSYR1 (Amano et al., 2007). PSKR1 interacts with the well-known co-receptor BAK1 in PSK signaling (Ladwig et al., 2015). Next to the control of root growth the interaction of PSK with its receptors is involved in hypocotyl cell expansion control (Stührwohldt et al., 2011). Moreover, a role for PSKRs as negative regulators of innate immunity has been proposed (Mosher et al., 2013; Tang et al., 2017). The *pskr1* mutant showed enhanced resistance against the hemibiotrophic pathogen *Pseudomonas syringae* as well as increased defense responses following elf18 application (Igarashi et al., 2012).

3.3. Plant innate immunity

In contrast to higher animals, plants do not possess mobile cells as part of their immune system. The mobile immune system is integrated by specialized cells, which detect and destroy enemies. The plant immune system however, is supposed to be present in every single cell and needs to allow each one of those to detect danger. In order to induce an effective defense response and to initiate signal cascades to alert parts of the plant about an imminent attack (Schilmiller and Howe, 2005; Jones and Dangl, 2006b). The plant immune system was shaped by millions of years of coevolution between plants and the corresponding pathogens. This convergent evolution results in a tremendous complexity at the molecular level (Asai and Shirasu, 2015). While the host plant is defending like a fortified castle, the invading pathogens developed special weapons to conquer the established walls of the plant immune system. The first layer of defense and therefore the “watch towers” of the plant defense can be found in the cell surface in form of pattern-recognition receptors (PRR). These receptors detect broadly conserved pathogen molecules (pathogen/ microbe-associated molecular patterns, PAMP/MAMPs). This system is known as PAMP- (or pattern)- triggered immunity (PTI) or sometimes also referred as MTI (MAMP- triggered immunity). A weapon of pathogens to overcome the first layer of defense are effectors. Effectors are targeted to the plant apoplast or cytoplasm. However, also against these weapons plants developed specific defense mechanisms. Effectors are recognized by receptors called Resistance (*R*) proteins, which recognize the presence of pathogen effectors directly or indirectly and induce a strong counter attack. This mechanism is known as effector-triggered immunity (ETI) (Jones and Dangl, 2006b).

3.3.1. They reveal themselves - the first layer of defense

The first layer of defense must be a broad system since plants are under continuous attack from various pathogens. Plants need to be able to detect a large diversity of different pathogens. The plant PRRs can recognize highly conserved structures common amongst numerous classes of microbes. Most of these structures are vital for the microbial life style and therefore underlie a negative selection pressure which is altered by the microbes to overcome the recognition (Boller and Felix, 2009; Monaghan and Zipfel, 2012). PTI is

therefore discussed as an evolutionary old defense system, which is strengthened by the fact that many PRRs are conserved amongst higher plants (Boller and Felix, 2009). The first layer of defense is established by the recognition of MAMPs by PRRs.

3.3.2. Recognize the enemy – pattern recognition receptors (PRRs)

The innate immunity system of multicellular organisms requires the acquisition of cell surface receptors that can differ between “self” and “non-self” molecules. This is achieved by the large family of pattern recognition receptors (PRRs). PRRs are capable of activating one or more signaling pathways. Microbes are often found in the plant apoplast and therefore separated from the plant cell interior. To sense environmental information across the plasma membrane in a selective manner, plants use families of plasma membrane- localized PRRs. Therefore, PRRs transduce the signal of danger into intracellular signals (Macho and Zipfel, 2014; Zipfel, 2014; Tang et al., 2017). PRRs can be broadly categorized into receptor-like kinases (RLKs) and receptor-like proteins (RLPs). The structure of RLKs consists of an extracellular receptor domain, a membrane-spanning domain and an intracellular kinase domain. This last domain is missing in RLPs (Morillo and Tax, 2006; Toer et al., 2009; Ben Khaled et al., 2015; Boutrot and Zipfel, 2017). In the Arabidopsis genome, more than 600 RLKs have been identified and were associated with numerous different signaling pathways and responses (Shiu and Bleecker, 2001; Shiu et al., 2004). This large family can be separated into two classes (Shiu and Bleecker, 2003; Tör et al., 2009). The first one includes RD kinases containing a conserved arginine (R) residue in front of an aspartate (D), which provides the catalytic activity and is important for the function as a kinase (Schwessinger et al., 2011). The second class are non-RD kinases, these kinases lack these two specific amino acids. To achieve the initiation and amplification of phosphorylation signals non-RD kinases may require a co-receptor (Dardick et al., 2012). A key characteristic for RLKs is their extracellular domain which determines their classification into further classes like leucine-rich repeat (LRR), epidermal growth factor (EGF)-like, lysine-motif (LysM) or leucine motif. Whereas, RLK-LRR are the receptors for peptide ligands such as MAMPs and DAMPs (Chinchilla et al., 2006; Yamaguchi et al., 2006; Zipfel et al., 2006). The second class of PRRs are the RLPs. They bind their ligands at the ectodomain but lack the intracellular kinase domain. Therefore, they require to bind the assembly with a co-receptor upon ligand binding in order to transduce the signal (Zipfel,

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2014). Various defense-associated, physiological and developmental functions rely on PRRs (Shiu and Bleecker, 2001; ten Hove et al., 2011; Araya et al., 2014).

The most well-studied PRR in plants is the receptor for the bacterial flagellin FLS2 (Chinchilla et al., 2006). FLS2 is the analogue of the mammalian TLR5 and perception and defense responses caused by the perception of flg₂₂ by these receptors have been well studied in plants and mammals (Fliegmann and Felix, 2016; Hajam et al., 2017). The extensive search for FLS2 orthologues lead to the identification of FLS2 orthologues in tomato, rice, grapevine and tobacco (Hann and Rathjen, 2007; Robatzek et al., 2007; Takai et al., 2008; Trdá et al., 2014).

PRRs with a non-RD kinase domain have been shown to interact with a RD receptor kinase. The RD receptor kinase functions as a co-receptor upon ligand binding. One of the best characterized co-receptors is the LRR-RLK BRI1-ASSOCIATED KINASE 1 (BAK1). BAK1 is a member of the SERK family and interacts with BRI1 to regulate brassinosteroid signaling. AtBAK1 form ligand-dependent heteromeric complexes with several defense inducing signals (Chinchilla et al., 2007; Roux et al., 2011; Schwessinger and Ronald, 2012; Jordá et al., 2016). The BAK1 mutants are drastically impaired in the perception of defense inducing signals.

A recent study in the field of PRR receptors could be a breakthrough in the research on receptor interactions. In the last years the number of identified and characterized receptor families increased continually. It is assumed that these receptors interact with each other. By investing of 40.000 potential extracellular domains of receptors and their interactions it was found that BAK1 interacts with a high number of receptors (Smakowska-Luzan et al., 2018). This research could help to uncover further receptor co-receptor complexes and their interactions.

3.3.3. It is time the defend – Effector-triggered immunity (ETI), the second layer of defense

The perception of a of a MAMP or DAMP and the induction of a PTI response is in general a very efficient defense system. However, certain pathogens or pathogen strains found a way to inhibit PTI responses. By introducing virulence effectors into the host pathogens developed a possibility to inhibit specific steps of pathogen detection or subsequent downstream signaling processes. This process is referred to as effector-triggered susceptibility (ETS) (Jones and Dangl, 2006b; Boller and He, 2009; Dodds and Rathjen, 2010). This mechanism is a crucial

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element of the host-pathogen coevolution cycle. Also, the host side developed a mechanism to defend against pathogen effectors by introducing a robust resistance response called effector-triggered immunity (ETI) (Jones and Dangl, 2006b; Jacob et al., 2013; Cui et al., 2015). ETI is famously also known as gene-for-gene resistance (Flor, 1971). This second layer of defense is based on a surveillance system detecting either directly secreted effectors or indirectly detecting a modified internal signal originating from the effector attack (Boller and He, 2009). This detection system is based on *resistance* (*R*) genes. These genes code mostly for intracellular NB-LRR proteins (Nucleotide Binding Proteins with Leucine-Rich Repeat domains) (Jones and Dangl, 2006b). The induced defense response by the perception of effectors by NB-LRR is regarded to be more rapidly induced, longer lasting and more severe than PTI. The ETI response culminates in the hypersensitive response (HR). HR is the apoptosis of the infected and the surrounding cells (Greenberg and Yao, 2004; Jones and Dangl, 2006b; Truman et al., 2006; Tsuda and Katagiri, 2010). The induction of HR response is under tight regulation. The HR signaling is strongly inhibited by PTI signaling, indicating a strict separation of the two layers of plant defense (Hatsugai et al., 2017).

Regarding the co-evolution of plants and their host, ETI is seen as the more dynamic process in comparison to PTI. ETI is often highly specific between a particular plant cultivar and a pathogen race (Dangl and Jones, 2001). Plants constantly adopt their *R* genes, while the pathogens change their effectors. This race of effector evolution and adaptation is known as the “arms race” and is nicely outlined in the zigzag model proposed by Jones and Dangl (2006) (Figure 2).

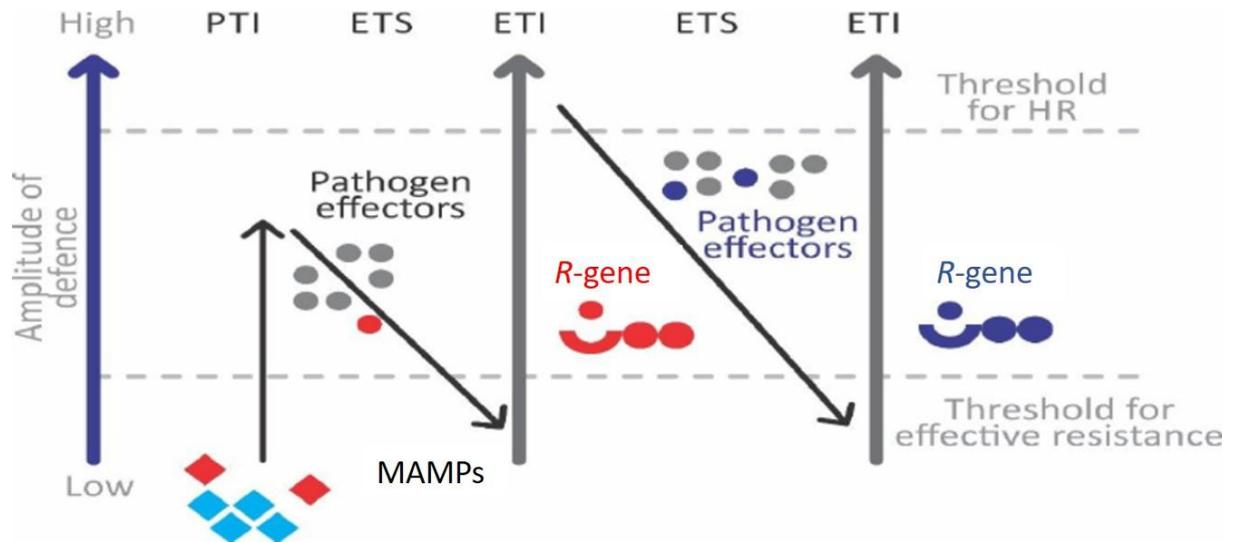


Figure 2: **The “zigzag” model describing the coevolution of plants defense mechanisms and the pathogen countermeasures.** The first level of plant defense is the induction of PTI. The detection of MAMPs induces these basal defense responses. Pathogens can evade from PTI or block its activation by carrying specific effectors that attenuate defense response and render the plant susceptibility to the pathogen (ETS). The recognition of pathogen effectors induces a strong defense response, stronger than the PTI response. This response leads to the cell-death like HR and finally to ETI. (adopted from (Jones and Dangl, 2006b))

3.4. Defense responses induced during PTI

Plants developed a physical barrier against pathogens that make them generally resistant against the majority of invaders. This barrier is termed non-host resistance and consist of the plant cuticle, the cell wall and constitutively produced antimicrobial compounds. All land plants protect their external surface of the aerial epidermis with a waxy cuticle (Osbourn, 1996; Somerville et al., 2004; Yeats and Rose, 2013). The detection of an elicitor induces a complex set of responses intended for resisting against a pathogen attack (Bigeard et al., 2015). Moreover, the perception of MAMPs and DAMPs triggers similar PTI responses (Yamaguchi and Huffaker, 2011). However, the kinetics and kind of induced defense response depends on the perceived elicitor. For example, the induction of protease inhibitors is specifically induced after perception of HAMPs (Herbivore associated molecular patterns) and some DAMPs but not MAMPs (Zebelo and Maffei, 2015; Zhu-Salzman and Zeng, 2015). The most common PTI responses will be discussed in the following paragraph.

3.4.1. Electrical signaling

The fastest defense response appears to take place at the only cell compartment that is in direct contact to the environment. At the plasma membrane a modulation of ion fluxes is among the earliest cellular responses to biotic and abiotic stresses (Ebel and Mithöfer, 1998; Shabala et al., 2006; Fürstenberg-Hägg et al., 2013). In form of an altered membrane potential a signal can travel through the plant. The generated action potential-like signal serve as a systemic defense signal (Maffei and Bossi, 2006). This signal on the cell surface can propagate with a speed of up to 40 m sec^{-1} (Volkov and Brown, 2006). A strong membrane depolarization was observed induced by the MAMPs elf18 and flg22. The depolarization was induced within 1-5 minutes after elicitor treatment and lasted for around 1 to 1.5 hours (Jeworutzki et al., 2010)

3.4.2. Ion fluxes

Upon elicitor perception plasma membrane channels are opened within 1-2 min. This causes a strong increase of intracellular Ca^{2+} and H^+ (Lecourieux et al., 2002). On the other hand the elicitor perception also causes a anion efflux (Boller, 1995). The strongly increased Ca^{2+} concentration is of particular interest since Ca^{2+} is known to function as second messenger in various cellular processes (Lecourieux et al., 2006). Moreover, innate immunity is regulated by calcium-dependent protein kinases (CDPKs) and act as sensor for Ca^{2+} (Boudsocq et al., 2010). Also, other defense responses are regulated by CDPKs. The production of reactive oxygen species (ROS) is regulated by phosphorylation of the ROS producing enzyme NADPH oxidase (Kobayashi et al., 2007).

3.4.3. Oxidative burst

One of the fastest defense response is the burst of reactive oxygen species (Torres et al., 2006). The plasma membrane-localized NADPH oxidase (RbohD) is mainly responsible for the ROS burst. This enzyme produces membrane-impermeable superoxide ($\text{O}_2^{\cdot-}$), which is converted into hydrogen peroxide (H_2O_2) in the apoplast (Liu and He, 2016). It was shown that the aquaporin AtPIP1;4 is crucial for the transport of H_2O_2 across the plasma membrane into the cytoplasm and therefore important for defense response induction (Tian et al., 2016). The plant is capable to produce tremendous amounts of ROS, which directly inhibit the pathogen

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or herbivore growth (Apel and Hirt, 2004). The plasma membrane-associated cytoplasmic kinase BIK1 interacts and phosphorylates RbohD upon elicitor perception (Li et al., 2014). Furthermore, by triggering the synthesis of SA and MAPK activation, ROS serves as a second messenger (O'Brien et al., 2012).

3.4.4. Changes in protein phosphorylation and MAPK activation

External stimuli are transduced into intracellular responses by the activation of MITOGEN-ACTIVATED PROTEIN KINASES (MAPK). This cascade starts with the phosphorylation of a MAP Kinase Kinase Kinase (MAPKKK), which phosphorylates a MAPKK, which in turn phosphorylates MAPK. These phosphorylated MAPK have various protein targets in the nucleus and the cytoplasm (Meng and Zhang, 2013). Regarding defense responses, MAPK cascades are activated after MAMPs and DAMPs perception. The Phosphorylation and activation of MPK3 and MPK6 is used as an assay for defense signaling (Asai et al., 2008; Rodriguez et al., 2010; Galletti et al., 2011).

3.4.5. Callose deposition

One of the later defense responses in the deposition of callose. In Arabidopsis, callose deposition can be detected from 16h onwards after MAMP treatment by fixing and staining the tissue with aniline blue (Gomez-Gomez et al., 1999). In the leaf tissue callose is located in the papillae, a tissue that stretches from the plasma membrane to the cell wall. However, the exact role of callose deposition in plant defense remains unclear. Callose deposition goes hand in hand with that of ROS, phenolic compounds and several cell wall proteins. These events have confirmed antimicrobial or cell wall reinforcing functions (Voigt, 2014).

3.4.6. Transcriptional changes

Upon pathogen invasion as well as perception of MAMPs and DAMPs plants launch a profound and dynamic reprogramming of gene expression. Nearly all previously described defense responses are regulated by a set of different genes (Tsuda and Somssich, 2015; Li et al., 2016). Studies of Arabidopsis revealed that already after 30min after flg22 treatment, expression of roughly 1000 genes were up regulated and only 200 genes down regulated (Zipfel et al., 2004). Among the induced genes are also the PRR FLS2 and EFR. These results

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reveal a positive feedback loop of PTI activation (Shiu et al., 2004; Zipfel et al., 2004). Comparative expression analysis of gene expression after infection with virulent *Pseudomonas* bacteria and a nonpathogenic strain showed that transcriptional response to the conserved bacterial patterns starts before the bacteria itself starts to multiply. The earliest induced genes are related to defense responses and salicylic acid (SA) biosynthesis. Genes involved in photosynthesis are down regulated. This indicates that resources are shifted towards the limitation of pathogen growth (Lewis et al., 2015).

3.4.7. Inhibition of seedling growth

The addition of MAMPs and DAMPs to the seedling growth medium leads to a strong inhibition of growth in a concentration dependent manner. This effect depends on the interaction of the elicitor and its receptor (Krol et al., 2010). The exact molecular mechanism behind the arrested seedling growth remains unclear. One possible explanation would be the shift of resources from growth towards defense mechanism (Walters and Heil, 2007; Boller and Felix, 2009).

3.4.8. Hormonal integration of immune responses

Several classical plant hormones are shown to be involved in defense responses. Among them jasmonic acid (JA), and salicylic acid (SA) are the major defense-related phytohormones. Moreover, other hormones are involved namely, ethylene, abscisic acid, auxin, gibberellins, cytokinins and brassinosteroids (Shigenaga and Argueso, 2016). In general, JA and SA are positive regulator of plant defense. JA regulates immunity against necrotrophic pathogens and SA immunity against biotrophic pathogens (Pieterse et al., 2009; Berens et al., 2017).

The fatty acid derivate JA has been reported to be an important downstream signaling upon necrotrophic pathogen attacks. JA levels increase locally after tissue damage (Wasternack, 2007; Bari and Jones, 2009). The active form of JA, JA-Ile is perceived by coronatine-insensitive 1 (*COI1*) This perception regulates a group of MYC transcription factors in initialize transcriptional reprogramming (Wasternack and Hause, 2013). Differently regulated genes are defensins, proteins with antimicrobial and enzyme inhibitory functions and marker genes for JA-dependent defense signaling (Manners et al., 1998). JA application triggers immunity against necrotrophic pathogens in *Arabidopsis* but also in rice and *Medicago truncatula* as

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well as a systemic priming of defense responses which is referred as induced systemic resistance (ISR) (Bostock, 2005; Schilmiller and Howe, 2005; Taheri and Tarighi, 2010; Berens et al., 2017).

A pathogen infection causes increased accumulation of SA in various eudicots while exogenous application of SA or its analogues triggers immune responses and resistance against pathogens (Berens et al., 2017). SA is perceived by a receptor complex containing several NPR (non expressor of PR) proteins (Yan and Dong, 2014). This signaling acts as activator of a large set of defense-related genes. These genes are referred to as pathogenesis-related (PR) genes (Dong, 2004). PR genes are diverse however several of these genes are shown the code for proteins with direct anti-microbial activity (van Loon et al., 2006). Moreover, the activation of PR genes at the site of infection often leads to similar responses in distal tissue. This tissue activates defense responses in order to be in a “ready state” to an imminent pathogen threat. This effect is called systemic acquired resistance (SAR) (Conrath, 2006; Vlot et al., 2008). However, SA seems not to serve as a mobile signal *per se* inducing immunity in uninfected tissue while several other molecules have been proposed to fulfill such a role (Shah and Zeier, 2013). An overview of the chronological induction of PTI responses is given in the Figure 3.

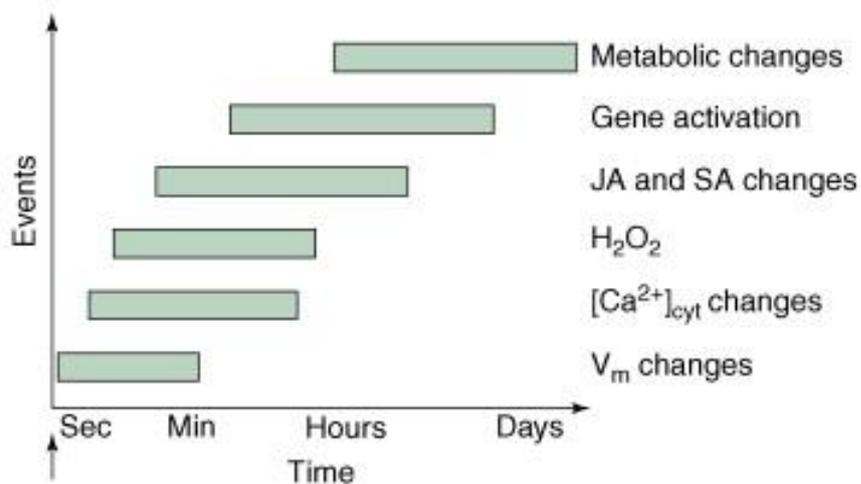


Figure 3: **The chronology of PTI responses.** After elicitor application various plant cell responses can be measured. Altered membrane potentials (V_m) and ion fluxes are the earliest events. The next responses are related to production of defense molecules and messenger (JA, SA and ROS/ H_2O_2). Finally, metabolic changes can lead to profound defense responses and long-lasting adaptations. (adopted from (Maffei et al., 2007))

3.5. Systemic acquired resistance (SAR)

Already in the year 1933 the concept of a acquired immunity in order to increase resistance upon a reinfection in plants was postulated (Chester, 1933). The term SAR was first proposed 30 years later upon work on tobacco. When the three lower leaves of a tobacco plant were infected with *Tobacco mosaic virus* (TMV) the upper leaves developed much weaker infection symptoms after a second infection 7 day subsequent to the first infection (Ross, 1961; Klessig et al., 2018). SAR is a complex mechanism of induced defense that can lead to a long-lasting resistance against a broad spectrum of unrelated pathogens (Durrant and Dong, 2004). Upon a local infection a transport of defense signals throughout the plant is initiated. These signals are generated and transported through the phloem via the apoplast to the uninfected distal tissue (Tuzun and Kuć, 1985; Gao et al., 2015; Singh et al., 2017). The nature of the systemic signal has not yet been uncovered however, in the last years at least 13 different possible signals have been proposed (Gao et al., 2015). One of the first proposed signals was SA. Indeed, SA, methyl SA (MeSA) as well as the accumulation of PR transcripts is required for SAR (Mishina and Zeier, 2007). Arabidopsis mutants unable to accumulate SA do not acquire systemic resistance upon infection with necrotizing pathogens while Arabidopsis plants overproducing SA show enhanced defense to pathogens (Conrath, 2006). PR gene accumulation is often seen as the molecular basis of SAR. Some of the typical SAR-induced PR genes code for enzymes possible able to hydrolyze microbial cell wall components (Van Loon and Van Strien, 1999). However, the accumulation of PR proteins does not *per se* explain the SAR phenomenon (Conrath, 2006). A key component for SAR is NPR1 (NON-EXPRESSOR OF PR1). NPR1 is an important downstream signaling element of SA. Overexpression of NPR1 leads to stronger PR gene expression after pathogen infection and a strongly enhanced disease resistance (Cao et al., 1998; Friedrich et al., 2001). SA can directly bind to NPR1 which leads to its monomerization (Wu et al., 2012). NPR1 monomers are transported to the nucleus and directly interact with basic leucine zipper transcription factors (bZIP) in order to activate defense gene expression (Gao et al., 2015; Birkenbihl et al., 2017). Another hallmark of SAR is the priming of defense responses whereas SAR-dependent priming is associated with faster and stronger defense responses to a secondary infection (Martinez-Medina et al., 2016)

3.5.1. Synthetic SAR activators

Since it was discovered that SA is an endogenous signal for SAR activation synthetic chemicals able to mimic SA and SAR induction have been identified. These synthetic elicitors are often termed plant activators and they trigger defense reactions and an induction of defense responses by mimicking interactions of natural elicitors or defense signaling molecules (Bektas and Eulgem, 2014). One of the first identified synthetic elicitors is polyacrylic acid and was shown to induce resistance of tobacco against TMV and to activate *PR1* gene expression (Gianinazzi and Kassanis, 1974). The first indication that exogenous application of SA contributes to the establishment of SAR is given by treatments with acetylsalicylic acid (Aspirin). Aspirin treatment causes induced resistance against TMV of tobacco by PR protein accumulation (White, 1979). This discovery paved the way to the discovery of more potent plant defense activators that are more suitable in crop protection. Two have the first discovered ones that are still widely used compounds are 2,6-dichloro-isonicotinic acid (INA), Beta-aminobutyric acid (BABA) and (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester (BTH) (Metraux et al., 1990; Ward et al., 1991; Görlach et al., 1996; Baccelli and Mauch-Mani, 2016).

INA was discovered by Ciba-Geigy and was shown to promote activation of defense in cucumber against fungal pathogens. Moreover, the resistance was also achieved in distal tissue indicating an activation of SAR (Metraux et al., 1991). INA provides also resistance against pathogens in pear, pepper and rice as well as in laboratory experiments tobacco and Arabidopsis (Metraux et al., 1991; Ward et al., 1991). However, INA does not trigger any changes in plant SA levels (Delaney et al., 1994) but has been reported to mimic biochemical and physiological effects of SA such as inhibition of catalase and the induction of cellular H₂O₂ accumulation (Conrath et al., 1995; Bektas and Eulgem, 2014). INA-induced *PR* gene expression is blocked in the *npr1* mutant this strengthens the role of INA in SAR (Wang et al., 2006). However, INA has phytotoxic side effects in crops and is therefore it has never been commercialized as agrochemical but is still used as tool to study SAR (Oostendorp et al., 2001).

BABA induces resistance against a very high number of stresses such as attacks by viruses, bacteria, fungi, oomycetes, nematodes and arthropods and also abiotic stresses like heat, cold and salt stresses (Balmer et al., 2015). After spraying of Arabidopsis plants with BABA, BABA get transported inside the plant whereas the young plant tissue act as sink (Jakab et al., 2001). Also, BABA was shown to boost SA pathway by enhanced *PR1* gene expression (Zimmerli et

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al., 2000). The enhanced defense response of BABA treated plants is explained by enhanced callose deposition and ROS burst (Ton and Mauch-Mani, 2004; Flors et al., 2008; Pastor et al., 2013). BABA induced drought and salt stress is regulated by interference with abscisic acid (ABA) (Zimmerli et al., 2007). Moreover, drought stressed wheat plants have been shown to have a reduced water use and therefore increased desiccation tolerance upon BABA treatment (Du et al., 2012). Another indication that BABA is a multifaceted synthetic elicitor is given by the observations that BABA boosts hormonal signaling pathways and the plant responses to the hormones (Flors et al., 2008)

BTH (benzothiadiazole) also known as acibenzolar-S-methyl ester (ASM) is a potent inducer of plant immune responses (Oostendorp et al., 2001). Until now BTH has been tested in more than 120 pathosystems (Faize and Faize, 2018). In Europe BTH is commercialized as “Bion”. Application of BTH has been shown to control downey mildew infections in vegetables and to control a range of fungal, bacterial and viral diseases of important crops like tomato, cucumber, broccoli, tobacco, melon, pear and apple trees (Scarponi et al., 2001; Zavareh et al., 2004; Pajot and Silué, 2005; Jiang et al., 2008). In apple it was shown that application controls fire blight, which is caused by the bacterium *Erwinia amylovora* (Brisset et al., 2000; Maxson-Stein et al., 2002). BTH itself does not show any direct effect on plant pathogens and is therefore not antimicrobial (Friedrich et al., 2003). BTH is a functional analogue of SA since it induces the same SA-characteristic expression profile (Friedrich et al., 2003). It was suggested that BTH is metabolized by the plant into acibenzolar by the enzyme SABP2. SABP2 silenced tobacco plants do not induce SAR typical gene expression while acibenzolar fully induced SAR in the same plants (Tripathi et al., 2010). One potent mechanism of BTH in order to prime plant defense is the potentiated activation of the defense associated MAPKs, MPK3 and MPK6. BTH induces the accumulation of the non-phosphorylated forms as well as an increased mRNA level of these two MAPK (Beckers et al., 2009). Moreover, BTH has the capacity to induce the expression of the important PRRs; FLS2, BAK1 and CERK1 (Tateda et al., 2014). This indicates that BTH primes defense responses at the receptor level and by mimicking SA signaling, also large sets of defense gene transcripts. This transcriptional response includes WRKY transcription factors. The genes *AtWRKY29*, *AtWRKY6*, *AtWRKY53* are strongly transcribed after stress application following pre-treatment with BTH. Moreover, BTH pre-treatment triggers several histone modifications that are found at actively transcript

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genes. *AtWRKY29* shows an elevated level of H3K4me3, H3K4me2, H3ac and H4ac and *AtWRKY6* as well as *AtWRKY53* enhanced levels of H3K4me3 and H3K4me2. These findings are strengthened by the observation that BTH-induced trimethylation of H3K4 is reduced in the *npr1* mutant (Jaskiewicz et al., 2011). Since BTH was commercialized in the year 1989 it was demonstrated several times that BTH application has positive effects on plant health (Faize and Faize, 2018). However, it was shown that about 75% of applied BTH was photolyzed within 4 hours on apple leaf tissue and only a small fraction undergo metabolism by the plant (Sleiman et al., 2017). This study reveals that even after the long time BTH is available on the market still numerous plant responses to this molecule remain to be discovered.

3.5.2. DAMPs as plant vaccines

In the last years the idea was raised that synthetic peptides could be replaced by DAMPs since they are capable to trigger immunity in a similar fashion compared synthetic peptides (Quintana-Rodriguez et al., 2018). Interestingly, it was assumed that plant and algal extracts, which contains many DAMPs, enhance the resistance against herbivore attacks in cabbage, tomato and maize. Moreover, algae extract lead to enhanced fungal and bacterial resistance in banana, apple, grapevine, melon, tomato, cucumber and carrot (Quintana-Rodriguez et al., 2018). For example, leaf extract of devil's trumpet elicits resistance against downy mildew in pearl millet (Devaiah et al., 2009). Seaweed extract has the capacity to enhance the resistance in cucumber plants to different fungal pathogens (Jayaraman et al., 2011). Resistance against herbivores and pathogens after an exposure with the plants own volatile organic compounds has been described in more than 30 plant species (Heil and Karban, 2010). Several volatile organic compounds can also have direct antimicrobial or insect-repellent effects (Quintana-Rodriguez et al., 2018). In summary, it seems that DAMPs could be a good tool to increase plant resistance in agriculture. Especially, extracellular fragments of DNA (eDNA) induces resistance in various plant species by increased formation of H₂O₂ and the activation of MAPKs (Duran-Flores and Heil, 2018). However, the increased "ready state" of the plant defense, which is referred as defense priming, was up to now not observed caused by other DAMPs apart of volatile organic compounds or crude plant extracts including eDNA. Indicating that, DAMPs as plant vaccines might not be the optimal choice for plant treatments.

3.6. Defense priming – The third layer of defense?

The ultimate achievement in plant immunity and maybe partly the compensation of a missing adaptative immune system, plants developed the capacity to memorize a previous stress by promoting a primed state of enhanced defense. Defense priming is established in the plants tissue exposed to a priming stimulus as well as systemically to unharmed, or untreated parts of the plant (Reimer-Michalski and Conrath, 2016). When plants are in a primed state, plants are capable of responding to very low stimulation with a faster and stronger defense response than unprimed plants. This effect has also been defined as systemic immunity or systemic stress tolerance. The effect of defense priming is also referred as plant sensitization and trained immunity (KuĆ, 1987; Ding et al., 2012; van der Meer et al., 2015). In general, a primed plant can show a modified set of responses in comparison to unprimed (naïve) plants. The possible mechanisms are summarized in Figure 4. Plants that have been primed by a priming stress, including treatments with chemical SAR inducer, DAMPs or any abiotic stress, are capable of responding faster to a subsequent triggering stress. Other possible effects of priming include a stronger and more sensitive response to a triggering stress. Whereas plants react to a lower threshold of the triggering stress. It is also possible that primed plants induce different gene network(s) in response to subsequent stress, which might be better adapted to the specific stress than it is the case for unprimed plants (Lämke and Bäurle, 2017). After the priming stimulus, the plant undergoes a period of stress memory (Stief et al., 2014). Priming involves the gathering of information. The duration of the priming memory may be in the range of several days to weeks and in some cases transgenerational (Mauch-Mani et al., 2017). Two mechanisms involved in defense priming have been proposed. The first is the accumulation of signaling or transcription factors and the second epigenetic changes that allow plants to memorize the “ready state” (Bruce et al., 2007).

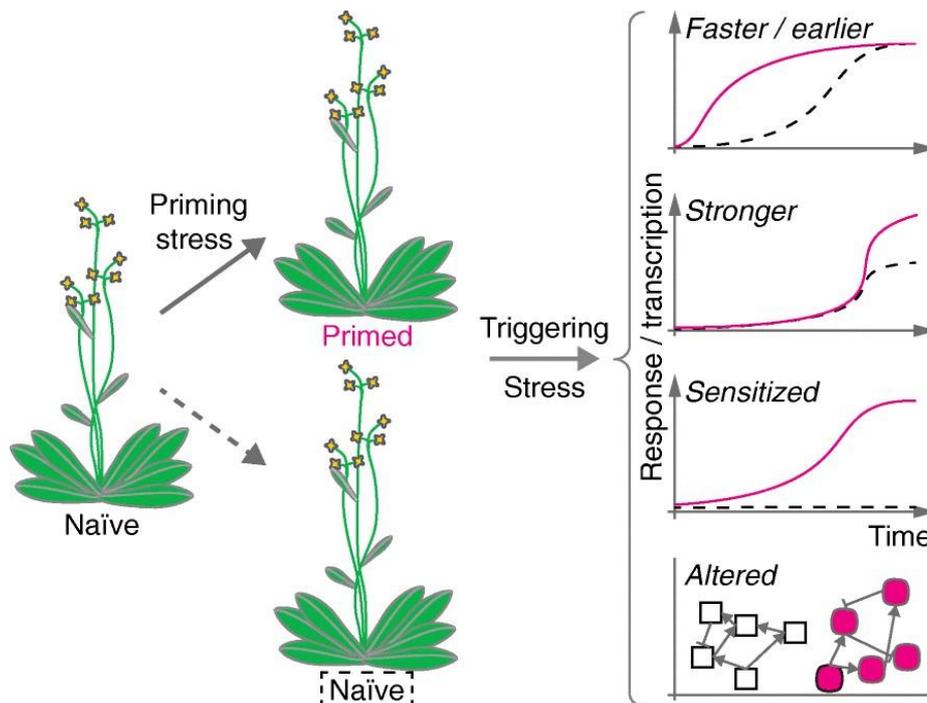


Figure 4: **Modified responses of primed plants in comparison to unprimed (naïve) plants.** Plants that faced a priming stress show different responses to a triggering stress than naïve plants. Primed plant responses are indicated in purple and unprimed responses in black. The primed plants may respond to the triggering stress faster/earlier, stronger or more sensitized than the unprimed plants. Primed plants may induce different networks of genes than unprimed plants. (adopted from (Lämke and Bäurle, 2017)).

3.6.1. *Transcriptional memory – one way to manifest stress priming*

It has been proposed that one layer of defense priming memory can be found at the transcriptional level. (Bruce et al., 2007). The priming stimulus can cause a continuous change in gene expression, although the plant is not facing the stimulus anymore. Whereas, the transcriptional modification includes an activation or repression as well as a modified transcriptional response, like a hyperinduction upon a second stimulus. Also, other typical transcriptional responses might contribute to priming memory. An induction of transcriptional feedback loops leading to the autoactivation of a transcription factor could take place after the initiative stress stimulus. Moreover, posttranscriptional modifications which influence the protein stability and a modification could contribute to an enhanced memory (Lämke and Bäurle, 2017).

3.6.2. Synthetic elicitors in defense priming

An enhanced capacity to react to a subsequent stress might be also established by an enhanced perception of a certain stress. Evidence for this post challenge prime state at the receptor level is given by treatments with the previously mentioned BTH. BTH treated plants show enhanced responsiveness to flagellin and chitin by increased expression levels of the receptors FLS2, CERK1 and the co receptor BAK1 (Tateda et al., 2014). Also, BABA has similar effects on plant receptors. The plasma membrane-localized and with FLS2 associated protein lectin receptor kinase VI.2 is required for BABA-induced resistance and is important for priming of MAMP-triggered immunity (Huang et al., 2014)

At the downstream level primed plants show the capacity of an increased ROS response upon a challenge. Priming treatments with SA, BTH and BABA lead to an enhanced ROS burst in response to a challenge with pathogens, MAMPs and DAMPs (Pastor et al., 2013; Tateda et al., 2014; Mauch-Mani et al., 2017). An interesting example of the enhanced primed resistance is the result that primed plants can block pathogen-mediated reopening of stomata during *Pseudomonas* infection. This led to the finding that BABA pre-treated plants might show drought stress tolerance mediated by the stomata closure (Camañes et al., 2012; Baccelli and Mauch-Mani, 2016). Furthermore, the previous described MAPKs also show enhanced activation in primed plants in response to pathogen challenge or biotic stresses (Beckers et al., 2009; Yi et al., 2015). Many other defense signals and transcription factors are activated by the initial triggering of the MAPK cascade (Conrath et al., 2015). In summary, priming at a transcriptional level is very multifaceted and affects all parts of plants metabolism and enables the plant to remember stressful situations for a limited time. However, epigenetic modifications enable plants to acquire memory and can cause long-term changes in gene responsiveness.

3.6.3. Epigenetic memory – the better way to manifest stress priming?

Epigenetic phenomena are widely spread among organisms and include genetic imprinting, paramutation, transposon activation, gene silencing and changes in the chromatin structure. Epigenetics was first defined in the year 1942 by Conrad Waddington as “The branch of biology which studies the causal interactions between genes and their products which bring the phenotype into being” (Waddington, 2012)(reprint). It is hypothesized that priming and

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the way a plant reacts to biotic and abiotic stresses relies strongly on epigenetic regulations (Bruce et al., 2007; van den Burg and Takken, 2009). It is assumed that the initial priming stimulus alters the chromatin structure or methylation level in such a way that gene promoters are more accessible and therefore facilitated to activate (Mauch-Mani et al., 2017; Ramirez-Prado et al., 2018).

DNA methylation is a widely studied epigenetic mechanism and is shown to be a dynamic regulatory mechanism of defense genes and stress priming. In plants, methylation of cytosine DNA base residues can have been classified in three sequence contexts, methylation can be separated in symmetric (CG, CHG) and asymmetric (CHH) DNA methylation patterns (where H is very base excepted of G). DNA methylation in all sequence contexts can be triggered by small interfering RNAs (siRNA) via a *de novo* methylation pathway termed RNA-directed DNA methylation (RdDM). The onset of RdDM begins with the production of RNAs by the Polymerase IV (Pol IV). The produced RNAs undergo several processing steps leading to the production of siRNAs that are loaded into ARGONAUTE 4 (AGO4). The formed complex has been proposed to base-pair with a nascent RNA scaffold which is produced by POL V. This complex is established the physical interaction of AGO4 with the large subunit of POL V, named NUCLEAR RNA POLYMERASE E1 (NRPE1). The methylation of DNA is finally established by the subsequent interaction with DOMAINS REARRANGED METHYLTRANSFERASE2 (DRM2). The second possible mechanism leading to DNA methylation is the POL II-RDR6-dependent RdDM pathway. Here, single stranded RNA (ssRNA) are transcribed by POL II and converted into double stranded RNA (dsRNA) by RNA-DEPENDENT RNA POLYMERASE 6 (RDR6) and then processed into 21-22 nucleotides (nt) long siRNA. These siRNA are loaded into AGO6 that can also interact with the scaffold RNA transcribed by POL V, which therefore establishes DNA methylation. The established DNA methylation marks in CG and CHG context are maintained through mitosis and meiosis via a pathway which is catalyzed by METHYLTRANSFERASE1 (MET1) and CHROMOMETHYLASE3 (CMT3), respectively (Espinás et al., 2016; Ramirez-Prado et al., 2018; Zhang et al., 2018). Factors involved in the establishment and maintenance of methylation have been found to control plant immunity and therefore provide evidence of epigenetic regulation of plant immunity. For instance, plants that are globally defective in maintaining CG and non-CG methylation in *met1-3* show enhanced defense responses when exposed to *Pseudomonas syringae* pv. *tomato* DC3000 (Downen et al., 2012). Moreover, it was

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shown that the global level of DNA methylation during defense response upon bacterial infection is reduced and that expression of defense-related genes is promoted by DNA hypomethylation during pathogen infection (Yu et al., 2013). Evidence that DNA methylation is part of defense priming was given by a study that showed a different capacity of hypermethylated and hypomethylated mutants to prime the activity of defense-related genes and callose deposition (López Sánchez et al., 2016). Moreover, another important factor for DNA methylation namely AGO4 is involved in immunity. The *ago4* mutant is more susceptible to *Pseudomonas* infection (Agorio and Vera, 2007).

The claim that “priming smells of epigenetics” is mainly based on findings involving histone modifications at defense-related genes (Waterborg, 2011). Histone modifications influence chromatin compaction and therefore the accessibility of genes for transcription as well as replication and recombination (Mauch-Mani et al., 2017). Modifications of histones are posttranslational and can, among others, consist of the addition of methyl or acetyl residues. Well characterized examples of these modification are acetylation of histone H3 at the lysine (K) 9 (H3K9ac). This histone mark is associated with the positive gene transcription activity. On the other hand, the histone mark H3K27me₃, which is a trimethylation of histone H3 at the lysine 27, is associated with repressed gene transcription activity (Pasini et al., 2008; Zhou et al., 2010). Especially, the histone mark H3K4me₃ is considered as a primary marker of stress memory (Conrath et al., 2015). It was shown that the maintenance of heat stress memory is mediated by H3K4 methylation at the side of a heat-inducible transcription factor (Lämke et al., 2016). Participation in defense priming was also shown in the case of histone acetyltransferases and deacetylases. The mutant *hac1-1* (histone acetyltransferase 1) is involved in bacterial resistance and defense priming following PTI reactions (Singh et al., 2014). It seems that HAC1 links repetitive stress responses activation to defense priming. In line with these results is the finding that knock out of RPD3/HDA1-class histone deacetylase (HDA19) results in a de-repression of SA-based defense (Choi et al., 2012). Moreover, the great majority of genes involved in SAR seems to be primed resulting by an interplay with different histone modifications (Conrath et al., 2015; Mauch-Mani et al., 2017). One report that shows concrete involvement of histone methylation of defense priming reported that treatment with BTH increases the appearance of H3K4me₂ and H3K4me₃ on three important defense-related transcription factors (*WRKY29*, *WRKY6*, *WRKY53*). BTH induced priming at

these genes showed a transcriptional memory after a lag phase of several days (Jaskiewicz et al., 2011). However, since also abiotic stress can induce defense priming (Singh et al., 2014) and defense inducing agents reduce between 20 and 85% of plant infection, it might be that plants in the field are already primed due to the various stresses they have to face continuously (Walters, 2009).

3.6.4. Transgenerational stress priming

The finding that certain DNA methylation patterns are inheritable paved the way to the hypothesis that some traits that are regulated by DNA methylation could be passed on to subsequent generations. Several studies showed an effect in the progeny of plants infected with tobacco mosaic virus or exposure to UV light or flg22 treatment. Progeny of plants infected with tobacco mosaic virus showed greater resistance while plants of which parents were exposed to UV or flg22 resulted in a greater homologous recombination frequency (Roberts, 1983; Molinier et al., 2006). Interestingly, also the chemical SAR activator BABA was shown to induce resistance in the progeny (Slaughter et al., 2012). After comparison of transgenerational resistance in RdDM mutants with wild type plants, it was suggested that transgenerational SAR is achieved through induced hypomethylation at non-CG DNA sites. (Luna et al., 2012; Luna and Ton, 2012). Next to the possibility of inherited DNA methylation marks, an alternative is that histone modifications are inherited through nucleosome recycling or the copying of modifications onto newly incorporated histones. This hypothesis is based on findings on the widely studied gene FLOWERING LOCUS C (*FLC*). This transcription factor acts as repressor of floral transition and is regulated by the histone mark H3K27me3. During embryogenesis the vernalized state of FLC is reset by the activity of an H3K27 demethylase (Crevillén et al., 2014). Mutants lacking the demethylase inherit the vernalization state to their offspring. Intergenerational stress memory was confirmed in a study on hyperosmotic stress priming. Plants which were stressed during their vegetative development passed on the stress memory for at least two generations. However, this stress memory was reset after one stress-free generation (Wibowo et al., 2016). Transgenerational epigenetic stress memory is meiotically stable and extends for at least one stress-free generation. One study showed that stress-dependent mobilization of retrotransposons and their directed integration in the genome can be stably inherited. This stable integration could

possibly lead to a more stress-resistant progeny (Thieme et al., 2017). This finding could open a door for the introduction of beneficial plant traits.

3.6.5. Memory is not for free – costs and omission of stress priming

Induced transgenerational resistance could possibly result in costs for the plant. On the level of hormonal regulation of plant defense, it was shown that the progeny of plants primed with a SA pathway inducing pathogen downregulate JA-dependent defense. This results in an increased susceptibility in these plants against JA-pathway dependent bacterial infections (Luna et al., 2012). Overall, defense priming is assumed to be beneficial for the plants with a generally positive cost-benefit balance in times of stresses. However, the advantage of a primed “ready state” becomes only obvious upon a subsequent exposure to a second stress, whereas a primed plant can outperform an unprimed plant. If this second stress is not accruing only the costs of priming influence the plants fitness. The activation and maintenance of the prime state of enhanced defense in form of the deposition of dormant signaling enzymes as well as the storage in form of epigenetic marks on defense gene promoters could result in fitness consequences (Conrath et al., 2015; Martinez-Medina et al., 2016). However, defense priming has lower costs than the direct activation of defense. In summary, research on the impact of defense priming on possibly negative effects is currently underrepresented. A possible model of the impact of defense priming on defense responses and plant fitness is summarized in Figure 5.

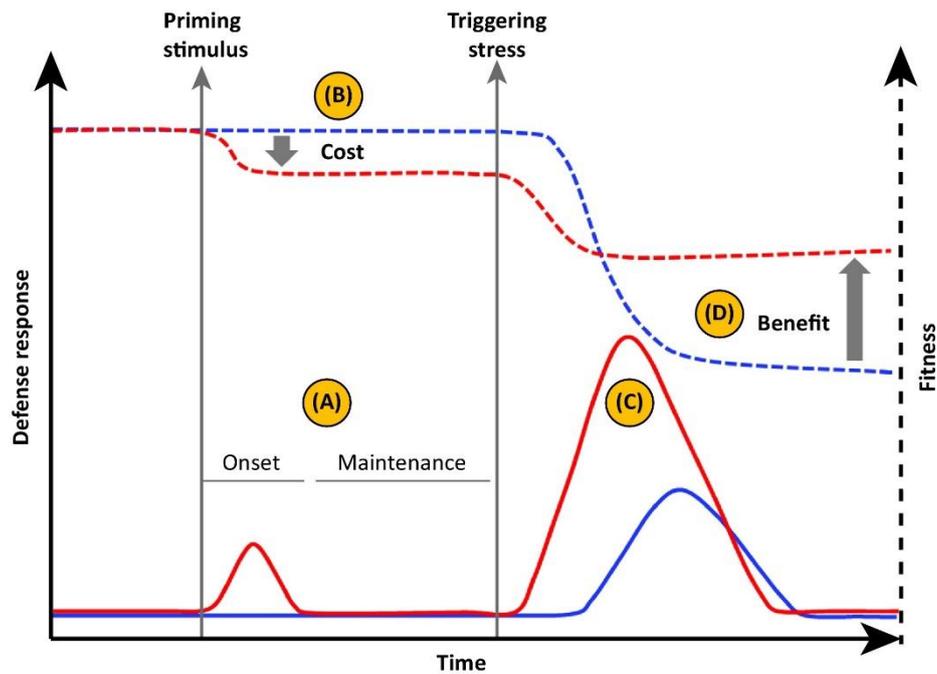


Figure 5: **Summary of the relation between defense responses (solid lines) and plant fitness (dashed lines) in primed (red) and unprimed plants (blue).** **A)** Defense reaction during priming and in the primed state is only transient and weakly induced. **B)** The direct activation of defense and the resulting fitness costs without priming stimulus is higher than the fitness cost of the priming stimulus. **C)** Upon a triggering stress, primed plants are capable of mobilizing cellular defense in a faster, earlier, stronger and more sustained fashion than unprimed plants. **D)** Primed plants do defend better against the triggering stress than unprimed plants. Adapted from (Martinez-Medina et al., 2016).

In the last years it became clear, that plants are also able to forget certain stresses. It might be an advantage for the plant to forget a previous stress in case the plant is sensing a false alarm signal (Crisp et al., 2016). In the case of a maladaptive effect of stress memory it is reasonable to consider possible mechanisms for resetting. A screen for factors involved in the erasure of epigenetic stress memory resulted in the identification of two genes. DECREASE IN DNA METHYLATION 1 (DDM1) and MORPHEUS' MOLECULE 1 (MOM1) are key factors to prevent transgenerational memory (Iwasaki and Paszkowski, 2014). Moreover, the role of RNA metabolism has great potential as a regulatory mechanism in memory resetting. Three mechanisms have been proposed to facilitate resetting of the transcriptome. At the transcriptional level are fast activation exonucleases (1) as well as miRNA silencing (2) and siRNA silencing (3) that could play a role in a directed resetting of gene expression (Crisp et al., 2016). However, not much is known how about how changes in mRNA stability influence transcriptional memory.

One research group showed a rather unusual memory response pattern by a subset of hydration stress response genes. Several exposures to hydration stresses has been assumed to prime the response to a subsequent hydration stress. However, it was found that a subset of genes responds to a first stress but then returns to a basic pre-stressed expression level during watered recovery and do not respond to subsequent stresses. The transcription factor MYC2 was identified to be a critical component for the memory behaviour of this subset of genes (Liu et al., 2014; Liu et al., 2016).

3.7. Antisense transcription

Next to the epigenetic regulation of gene expression another emerging regulation of transcription involves the expression of antisense transcripts. It has been previously reported that next to transcription of genes in sense they can also be transcribed in antisense orientation. Antisense transcripts include partial or complete sequences complementary to other transcripts and are endogenous RNA molecules (Wang et al., 2005). They play an important role in various processes, including the response to biotic and abiotic stresses (Terry and Rouzé, 2000). Antisense transcripts are widespread in both prokaryotes (Wagner and Simons, 1994) and eukaryotes (Vanhée-Brossollet and Vaquero, 1998). Moreover, evidence suggest that transcription of antisense RNAs have the potential to alter RNA processing, transport, stability and translation (Vanhée-Brossollet and Vaquero, 1998). Interestingly, antisense transcripts can modulate transcript levels via an RNA silencing mechanism. Double stranded RNAs emerging from sense and corresponding antisense transcripts could be processed into siRNA which then trigger silencing (Borsani et al., 2005; Wang et al., 2005). Moreover, it was shown that antisense transcripts are widely presents in apple. Notably, the percentage of antisense in apple is higher than that identified in annual plants like Arabidopsis (Celton et al., 2014).

3.8. The aims of this thesis.

The plant immune system is under complex genetic and epigenetic regulation and can be triggered by external as well as endogenous signaling peptides. At the beginning of my thesis the number of discovered and characterized small endogenous signaling peptides was constantly increasing. However, the number of potential small endogenous peptides in the Arabidopsis genome is tremendously high. In fact, the Arabidopsis genome harbors more than thousand genes that potentially code for secreted peptides (Lease and Walker, 2010). Apart from the induction and regulation of defense responses by small endogenous peptides, the plant is capable of remembering previous stresses by a mechanism referred as priming. At the beginning of my thesis the research focused mainly on the molecular mechanism of priming memory maintenance. Less published research focused on the plants capability to forget previous stresses. Thus, the overall focus of my thesis is to uncover to molecular mechanisms of induction, maintenance and omission of plant defense responses and memory by endogenous, exogenous and synthetic plant elicitors.

To achieve the first aim of my thesis I was working in close collaboration with the bioinformatic group at IRHS Angers. This group developed a bioinformatic pipeline to predict previously unidentified small endogenous plant peptide families. As a proof of concept, my goal was to characterize the properties and effects of the newly predicted small peptide family termed SCOOP (Serine riCh endOgenOus Peptide). The first very promising results opened the door for the discovery of various interesting effects of several small peptides of the SCOOP family. I show that the SCOOP family peptides are involved in defense responses and root development in Arabidopsis. My work on the SCOOP peptide family is described in the chapters 4 and 5.

The second aim of my thesis was to study the ability of plants to memorize treatments with defense priming elicitors as well as the influence of a subsequent exposure to exogenous elicitors. The goals here were (1) to uncover the long-term transcriptional memory a salicylic acid-related defense priming compound induce in a plant (2) to test if this memory is stable following a subsequent exogenous elicitor treatment. To demonstrate the generality of my findings I carried out experiment on Arabidopsis and apple. My work on the plant stress

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memory behavior and the loss of the primed state of transcription by a subsequent stress is described in chapter 6.

4. The SCOOP12 peptide regulates defense responses and root development in *Arabidopsis thaliana*.

The work described in this chapter was resulting from a collaboration between the bioinformatic group and me. A modified and extended version of this chapter was published in Gully et al., (2018) (Appendix in this thesis). The SCOOP peptide family regulates defense response and root development in *Arabidopsis thaliana*. My contribution was all experiments involving the application of peptides and certain pathogen infections (protection assay and pseudomonas infection of *proscoop12*) as well as creating a CRISPR-Cas9 mutant in the Col-0 background and data compilation. The phospholipid pathway activating capacity of SCOOP12 was investigated in a collaboration with the iEES-Paris.

4.1. Abstract

Small secreted peptides are important actors in plant development and stress response. Even though numerous *Arabidopsis thaliana* genes have the potential to produce these peptides, the vast majority of them have not yet been characterized for their biological functions. In this study, using a targeted *in silico* approach, we identified a small family of 14 Arabidopsis genes encoding precursors of Serine rich endogenous peptides (PROSCOOP). Plants defective in one of the family members (PROSCOOP12) show enhanced root growth. Searching through all Brassicaceae homologs of PROSCOOP12 we identified a conserved motif indicating that it encodes for a putative secreted peptide. The exogenous application of the synthetic peptide SCOOP12 induced various defense responses and pathogen tolerance in Arabidopsis. Our findings show that SCOOP12 has numerous properties of damage/danger-associated molecular pattern (DAMP) and is perceived in a BAK1 co-receptor dependent manner. In conclusion, we demonstrate that SCOOP12 fulfills all structural features of a post-translationally modified peptide that modulates defense responses and root elongation.

4.2. Introduction

Plants are facing continuous attacks by pathogens. To counter constant pathogen aggressions, plants have developed sophisticated perception and defense systems. These plant responses are regulated by complex networks involving regulatory proteins and hormones and are associated with massive changes in gene expression (Buscaill and Rivas, 2014). Among the involved actors, it has been shown that small secreted peptides, also named peptide phytohormones, play an important role through their direct interaction with pathogens or through their function in development and cell-to-cell communication involving ligand-receptor interactions (Murphy et al., 2012; Marmiroli and Maestri, 2014).

The secreted peptides derive from protein precursors having a shared N-terminal signal peptide which target the protein to the secretory pathway. They can be categorized into two major classes: (i) the small post-translationally modified peptides (PTMP) which are the targets of posttranslational maturations and are produced through a proteolytic processing and (ii) the cysteine-rich peptides (CRP) characterized by an even number of cysteine residues involved in intramolecular disulfide bonds (Tavormina et al., 2015). Although they are mainly involved in plant growth and development processes, it has been shown that numerous genes encoding secreted peptides are also involved in plant defense mechanisms (Albert, 2013). For instance, the CRP class includes the antimicrobial peptides (such as knottins and defensins) which interact and disrupt the pathogen cell membrane (Goyal and Mattoo, 2014). Regarding PTMPs, families such as the phytosulfokines, CLE/CLV3, IDA/IDL or PSY are actors in processes regulating a large panel of plant-pathogen interactions (Lee et al., 2011; Shen and Diener, 2013; Vie et al., 2015; Rodiuc et al., 2016).

Among secreted peptides, those showing immunity-inducing activity have been classified as damage/danger associated molecular pattern, *i.e.* DAMPs (Heil et al., 2012), by analogy with the exogenous microbe-associated molecular patterns, *i.e.* MAMPs (Boller and Felix, 2009). By using lytic enzymes, a pathogen can penetrate the plant cell wall and the cell wall fragments released into the apoplastic space can be perceived by neighboring cells, resulting in defense reactions. Oligalacturonides and cutin monomers are examples of DAMPs which

The SCOOP12 peptide regulates defense responses and root development

get released upon fungal infection (Fauth et al., 1998). Also, other molecules, which are not located in the extracellular space under normal conditions such as DNA, ATP and some sugar molecules can serve as DAMPs. Their perception by neighboring cells elicits innate immunity as well (De Lorenzo et al., 2011). The small peptide *AtPep1* is a well-documented DAMP (Bartels and Boller, 2015). A first induction of *AtPep1* and other peptides of this gene family by wounding or pathogen attack has a positive feedback on the expression of its own precursors as well as defense marker genes that is thought to amplify defense signaling pathways (Huffaker and Ryan, 2007).

However, only a small fraction of the gene space likely to encode signaling peptides has been described and their diversity appears to be largely underestimated (Matsubayashi, 2014). Indeed, the *Arabidopsis* genome contains more than a thousand genes harboring secreted peptide features for which the biological function is currently unknown (Lease and Walker, 2006, 2010). This lack of data can be explained by the fact that this type of genes has only recently been detected due to their small size and their low sequence conservation (Silverstein et al., 2007). Furthermore, the frequent functional redundancy inside these gene families (Matsubayashi, 2014) renders mutant knock-out approaches less successful. The mining of previously published transcriptomes is an efficient way to explore this unknown gene-space and decipher functions of new genes for which, without reference, the inference of function by similarity cannot be applied. Based on transcriptome meta-analysis and bioinformatics predictions in a 'guilt by association' approach, we identified a peptide family, whose one member at least is involved in plant immunity and root development.

This work describes the identification of a gene family specific to the Brassicaceae genus encoding putative secreted peptides. The functional characterization of *PROSCOOP12*, one of its members in *Arabidopsis*, shows that this small gene could act as moderator in the response to different pathogen aggressions and in root development presumably via controlling ROS detoxification. Based on the prediction of conserved motifs present in this family, we then illustrate that the small endogenous *SCOOP12* peptide displays most properties of a DAMP.

4.3. Results

4.3.1. Identification of the PROSCOOP gene family

As previously mentioned, the Arabidopsis genome harbors more than a thousand genes with properties of a potentially secreted peptide. Among them, one gene that we termed *PROSCOOP12* (AT5G44585 in TAIR10) caught the attention of the bioinformatic group at INRA Angers. This gene has an uncommon and highly informative transcription profile. By meta-analysis of the CATMA micro-array dataset (Gagnot et al., 2008) *PROSCOOP12* was found to be constitutively expressed in roots but strongly induced in leaves in response to a large panel of different biotic stresses. In roots *PROSCOOP12* shows a constitutive expression in normal growth condition but is down-regulated in numerous conditions affecting root elongation such as nitrogen starvation (Krapp et al., 2011). These observations led us to investigate the role of *PROSCOOP12* in root development in greater detail.

The screening of the Arabidopsis genome revealed that *PROSCOOP12* belongs to a small family of 14 unknown genes with a similar intron-exon structure (2 or 3 exons), encoding proteins ranging from 72 to 117 amino-acids (aa) with a N-terminal signal peptide, targeting proteins to the endoplasmic reticulum present in all members of the family. The genes of the *PROSCOOP* family are organized in two tandemly arrayed clusters on chromosomes 1 and 5 (Figure 6A). The largest 37 kb long gene cluster on chromosome 5 contains numerous vestiges of transposable elements (Helitron type) which could have impacted evolution of this family through local duplication events. Manual annotation revealed two additional yet non-annotated genes (*PROSCOOP2* and *PROSCOOP3*) located between AT5G44565 and AT5G44568. Both share significant similarities with the other clustered *PROSCOOP* genes. Our manual annotation also led to the correction of the structure of AT5G44570 (*PROSCOOP5*) in which an over-predicted 3' coding exon has been removed. The size of the proteins, the number and the organization of paralogs, the aa composition (with the absence of cysteine) and the presence of a signal peptide are common features shared by the PTMP families previously published (Matsubayashi, 2014). This newly identified family has been named SCOOP, for Serine riCh endOgenOs Peptide. Its members termed *PROSCOOP1* to 14 encode putative precursors of the mature SCOOP1 to 14 peptides.

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In order to identify divergent yet still conserved smaller regions (ranging from 6 to 12 aa), Sebastien Aubourg and his colleagues of the bioinformatic group used the MEME algorithm (Bailey et al., 2015), excluding full length alignments, on the 74 identified homologs. This sensitive approach allowed the identification of two significantly conserved 11 aa-long motifs (Figure **6B**). These motifs were good candidates for functional mature peptides following the putative proteolytic processing of the corresponding precursor. Indeed, both motifs are proline-, serine-, arginine- and glycine- rich, as in previously described PTMP families such as CLV3/CLE (Betsuyaku et al., 2011), IDA (Vie et al., 2015), PIP (Hou et al., 2014) and CEP (Roberts et al., 2013). Motif 1 is more ubiquitous than Motif 2 since it was detected in 72 sites (e-value of $9.8e-213$) compared to 39 sites (e-value of $3.4e-179$) out of the 74 PROSCOOP homologs (present in all *Brassicaceae* species). Therefore, we have focused our downstream analysis on motif 1 (Figure **6B**), named SCOOP thereafter.

The *SCOOP12* peptide regulates defense responses and root development

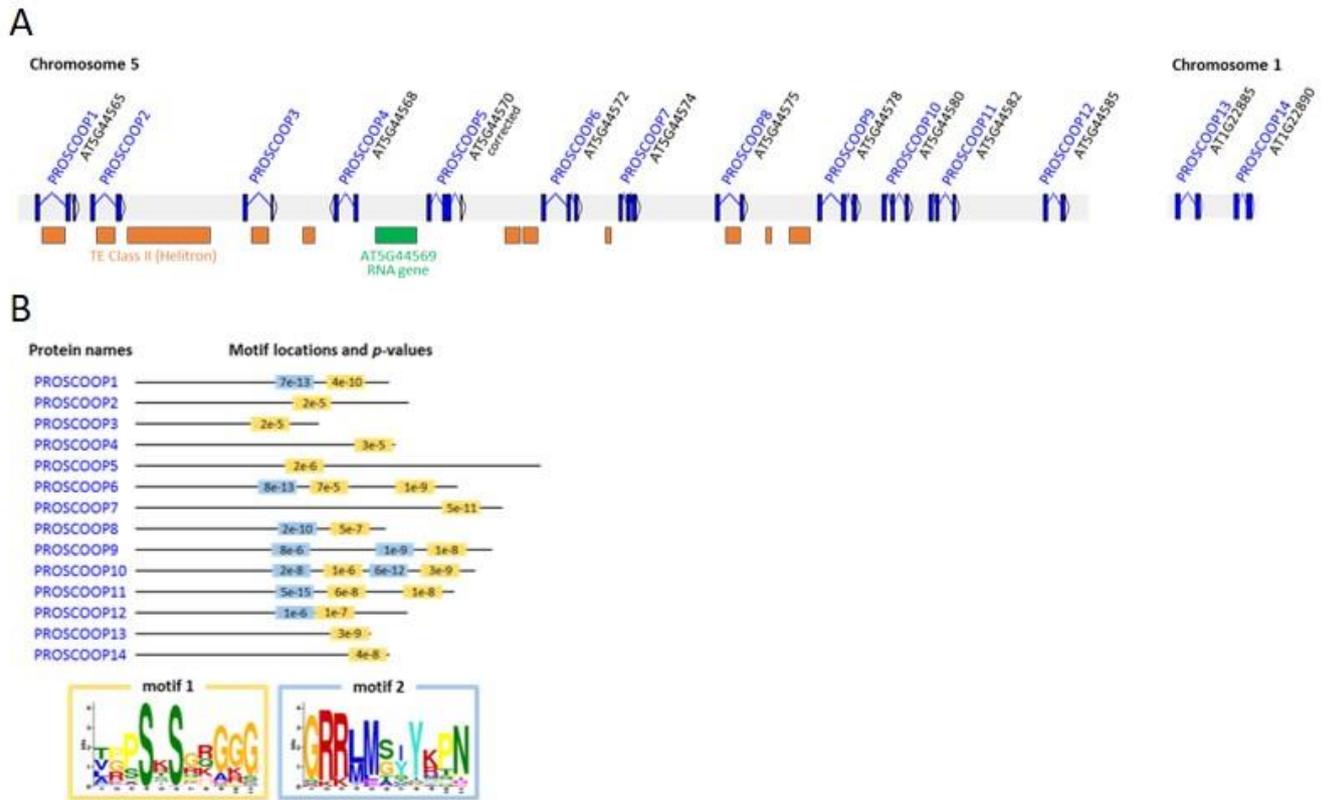


Figure 6: **A) *PROSCOOP* family gene organization:** coding exons and introns are represented by blue boxes and blue broken lines respectively. Remains of transposable elements (Helitron type) are represented by orange boxes and the green one indicates a putative non-coding RNA of unknown function. The TAIR gene names and corresponding *PROSCOOP* nomenclature are indicated. **B) Conserved motifs identified in the *PROSCOOP* family proteins:** The conserved motifs were found within all 74 homologous *PROSCOOP* proteins in Brassicaceae genomes. P-values and motif locations are only shown for the 14 members from Arabidopsis.

In order to assess the evolutionary conservation of the *PROSCOOP* family, an extensive search for homologs in GenBank was carried out. We identified this family in several Brassicaceae genomes reaching from *Eutrema salsugineum* to *Camelina sativa* and the number of identified homologs in these genomes ranged from 1 to 13. Outside Brassicaceae genus, no similar protein has been detected despite low stringency searches. The phylogenetic tree built from the multiple alignment of the 74 identified *PROSCOOP* homologs shows that tandem duplications occurred before speciation of the 8 different Brassicaceae species (Figure 7)

The SCOOP12 peptide regulates defense responses and root development

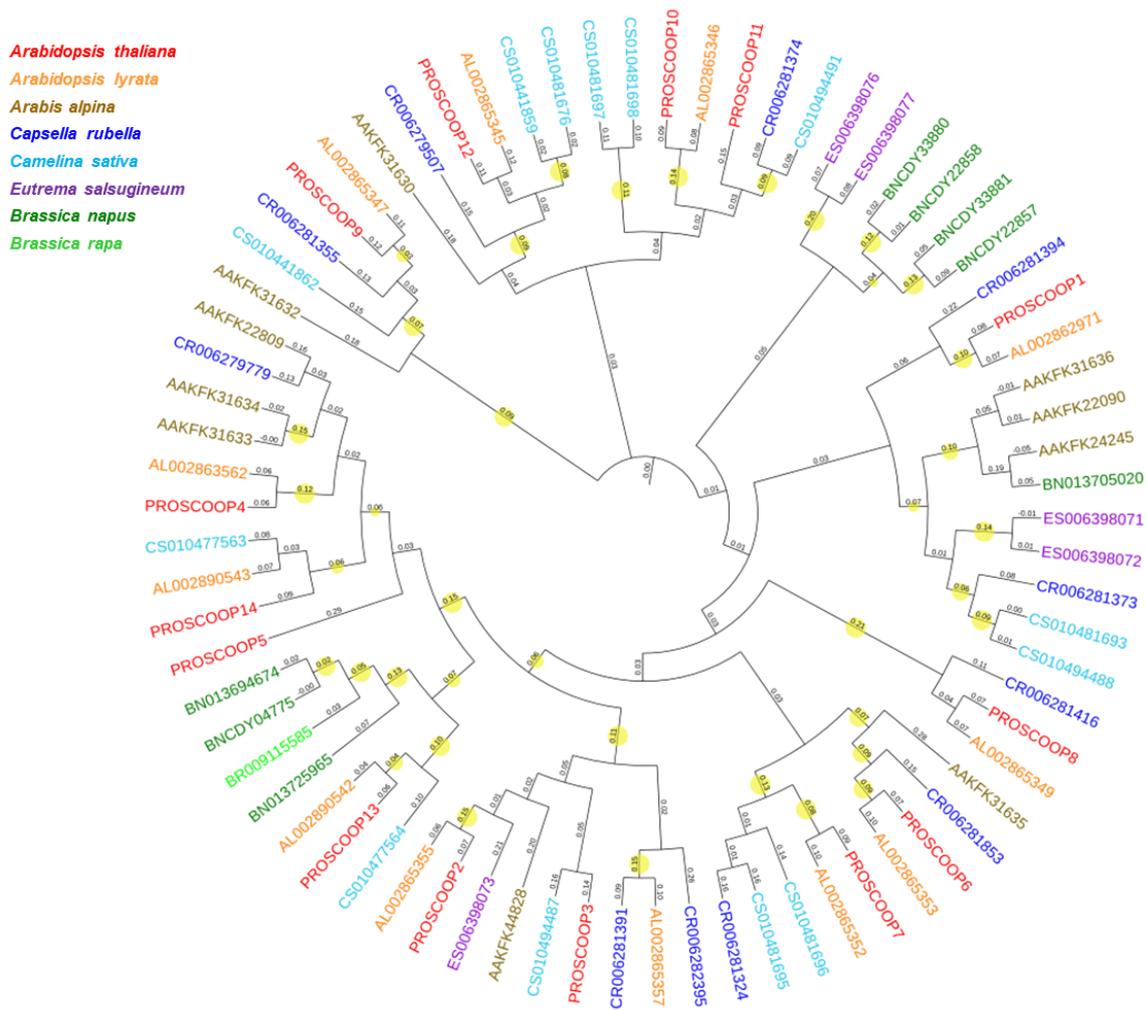


Figure 7: **Phylogenetic tree of PROSCOOP homologs.** The tree was built with the neighbor-joining method from the multiple alignment of 74 homologous Brassicaceae proteins. Gaps were ignored for tree building and 1000 bootstrap replicates were used to determine the robustness of each node (values > 50% are highlighted in yellow). Except for *Arabidopsis thaliana* for which PROSCOOP nomenclature is used, each protein is labelled with two letters (species) and its GenBank ID or XP number.

4.3.2. Use of the CRISPR-Cas9 system to generate proscoop12 in Col-0 background

While a T-DNA mutation in *PROSCOOP12* was available in the Wassilewskija (Ws) *Arabidopsis* accession, no mutation in that gene was available for Columbia (Col-0). In order to obtain a *PROSCOOP12* mutant in *Col-0* accession, we used the CRISPR-Cas9 approach to knock-out *PROSCOOP12* in this accession. A guide RNA (gRNA) was designed to target the first exon of *PROSCOOP12*. Following transformation *PROSCOOP12* was genotyped by sequencing for the

presence of mutations. In one line we detected a single alanine (A) insertion within the gRNA binding sequence (Figure 8A). The resulting frame shift led to an early stop codon only 10aa downstream the editing event. To confirm the sequencing result a 450 base pairs (bp) long PCR product was digested with the restriction enzyme *HaeII*, which enzyme recognition sequence (RGC GCY) is disrupted by the CRISPR-Cas9-targeted mutation in *PROSCOOP12*. The PCR product could not be digested in *proscop12* confirming the sequencing results (Figure 8B). In the previously mentioned T-DNA mutant *proscop12* in the Ws background *PROSCOOP12* was not transcribed (Figure 8C).

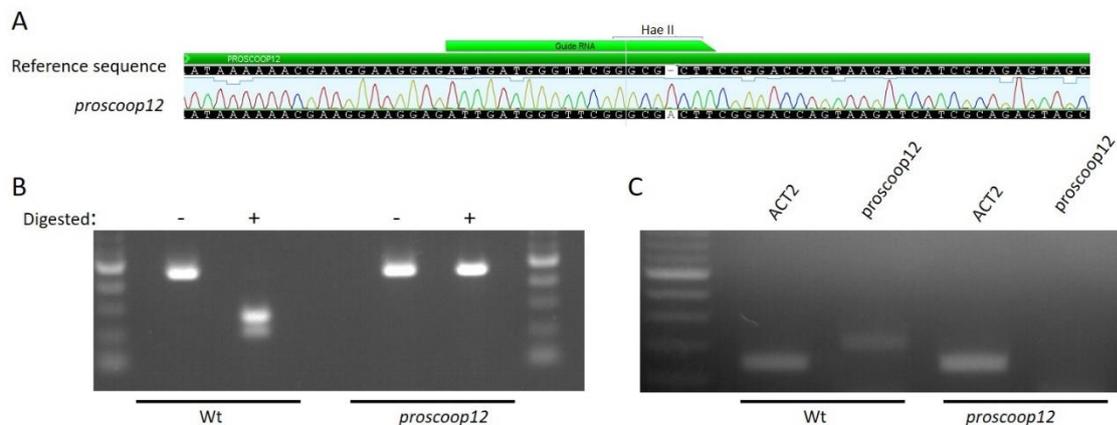


Figure 8: **Confirmation of mutations in *PROSCOOP12* in two accessions.** (A) The mutant in Col-0 (Columbia) background was created using the CRISPR-Cas9 approach. The guide-RNA was designed in the first exon. DNA of 15 *proscop12* mutants was extracted and sequenced. The alignment to the reference sequence (TAIR10) shows that *proscop12* has a single base insertion. (B) A 450bp long fragment covering the guide-RNA sequence was amplified by PCR. The PCR product of Wt and *proscop12* DNA was digested using the restriction enzyme *HaeII*. (C) Confirmation of absence of transcription of *PROSCOOP12* in the T-DNA knock-out line by RT-PCR in Ws (Wassilewskija). *Actin2* expression (*ACT2*) is used as control.

4.3.3. *PROSCOOP12* is involved in root development

Previous results on the *AtPep/PROPEP* peptide family, showed that this small peptide could play an important role in developmental processes. It was shown that *AtPep1* perception might inhibit root growth via regulation of *GLUTAMINE DUMPER (GDUs)* genes encoding amino acid exporters (Ma et al., 2014), and second a publication uncovered an acceleration of starvation-induced senescence upon Pep perception (Gully et al., 2015). Based on these findings and the transcriptomic analysis that suggested *PROSCOOP12* may play a role in root development, we compared the root length of wild type and *proscop12* plants. Indeed,

proscop12 plants developed significant longer roots than control plants in both accessions (Figure 9).

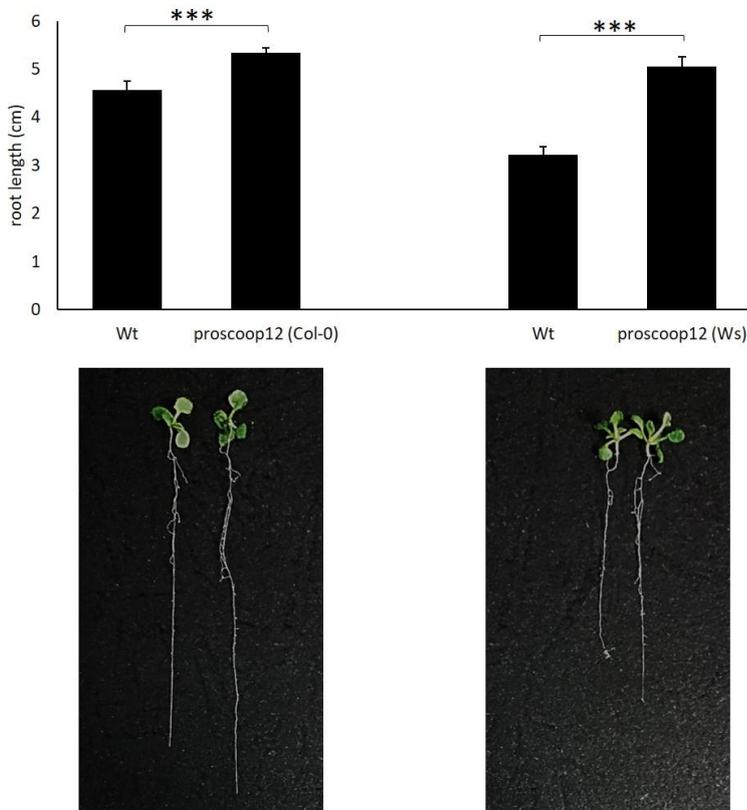


Figure 9: **phenotypic comparison between wild-type and *proscop12* plants.**

Root growth phenotypes were determined after 11 days. Wt plants were compared with *proscop12* in the two accessions *Col-0* and *Ws*. Student's t-test revealed that the different root length between wild-type and mutant is highly significant (***, $P < 0.001$).

4.3.4. The SCOOP12 peptide has the main features of DAMPs

The structural features of the PROSCOOP12 protein suggested that it should be classified as a secreted PTMP. However, at the functional level, its transcriptional behavior suggested that it may play a role as a DAMP. Indeed, the induction of *PROSCOOP12* expression by a large panel of biotic stresses and the root phenotypes identified in *proscop12* revealed some analogies with the *AtPROPEP1* and *AtPROPEP2* genes which encode for the precursors of the *AtPep1* peptide, a well-characterized DAMP (Bartels and Boller, 2015). Likewise, both genes are also induced by biotic stress (Huffaker et al., 2006) and the *AtPep1* DAMP is involved in root development since the overexpression of *PROPEP1* and *PROPEP2* resulted in significantly

longer roots (Huffaker et al., 2006). Therefore, we wanted to test if *PROSCOOP12* encodes for a peptide that may act as a DAMP and compare it to *AtPep1*

4.3.5. The SCOOP12 peptide induces immune responses in Arabidopsis

Based on the identification of the conserved motif 1 (Figure **6A**), a putative mature peptide SCOOP12 was defined (PVRSSQSSQAGGR) and synthesized in order to explore its biological function. Despite the non-predictable post-translational modifications of the mature peptide, we tested the exogenous application of the synthetic SCOOP12 peptide as previously described for the CLE and RGF PTMP families (Matsuzaki et al., 2010; Murphy et al., 2012; Whitford et al., 2012). Treatment of plants with SCOOP12 induced a wide range of long- and short-term immune responses (Figure **10**). One of the fastest defense responses is the production of ROS (Torres et al., 2006). We show here that SCOOP12 induced a more intensive ROS burst compared to *AtPep1* but weaker than *flg22* (Figure **10A**). Next, we wanted to study the effect of SCOOP12 on genes closely linked to early defense mechanisms. The *FLG22-INDUCED RECEPTOR-LIKE KINASE1 (FRK1)* has previously been shown to be induced by pathogens, pathogen-derived elicitors and salicylic acid through MAPK (mitogen-activated protein kinases)-mediated signaling (Asai et al., 2002; Boudsocq et al., 2010) and *AtPep1* (Flury et al., 2013). Therefore, we wanted to test if exposure to SCOOP12 could influence *FRK1* expression. Here, we measured *FRK1* steady-state transcript levels in detached leaves floating for 2h in solutions supplemented with SCOOP12 or *AtPep1*. Compared to controls, *AtPep1* and SCOOP12 treatments resulted in a 15-fold and 8.5-fold increase in *FRK1* expression, respectively (Figure **10B**). The deposition of callose is known to be triggered by conserved PAMPs (Luna et al., 2011) as well as DAMPs such as *AtPep1*. Callose staining after 24h of treatment with the elicitor peptides showed that SCOOP12 induced a callose deposition, yet at a weaker level compared to *flg22* or *AtPep1* (Figure **10C and 10D**). One of the long-lasting defense responses is an inhibition of growth caused by the elicitor. The addition of MAMPs or DAMPs to the medium can lead to a strong inhibition of seedling growth in a concentration-dependent manner, which is dependent on the receptor-MAMP interaction (Krol et al., 2010). Our results indicate that perception of SCOOP12 also lead to an arrest of growth. The effect is comparable to the *flg22* MAMP and the *AtPep1* DAMP (Figure **10 EFG**).

The SCOOP12 peptide regulates defense responses and root development

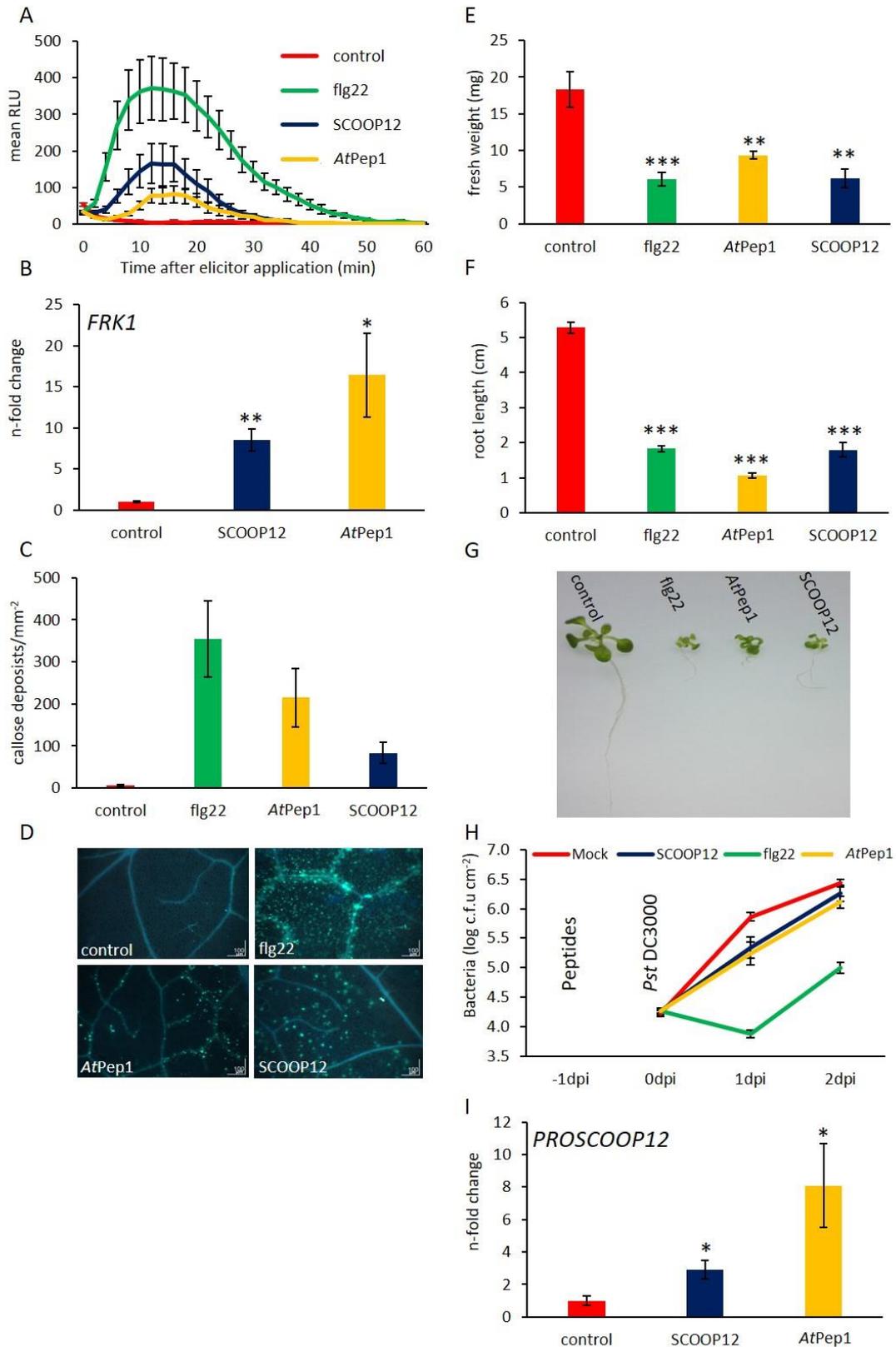


Figure 10: **Defense responses induced by SCOOP12.** (A) Reactive oxygen species (ROS) measured in RLU (relative light units) production in wild-type Arabidopsis leaf-discs (Col-0), treated with 1 μM for each peptide or without elicitor (control). Graphs display averages of 12 replicates. (B) Induction of (*FRK1*) gene transcription in soil-grown plants treated with 1 μM of the indicated peptide

The SCOOP12 peptide regulates defense responses and root development

or without elicitor (control). The bars represent the mean of three biological replicates. **(C)** Quantification of callose deposition. The bars represent the means of 4 replicates. **(D)** Localization of callose deposition by aniline blue staining. **(E-G)** Quantification of seedling growth inhibition. 5 days old seedlings were transferred from solid MS medium to liquid medium supplied with the indicated elicitors (all applied in a final concentration of 1 μ M) and were grown for additional 8 days before fresh weight and root length was quantified and pictures were taken. For all experiments: error bars show \pm SE of the mean. **(H)** Arabidopsis wild-type (Col-0) plants were pre-treated for 24h by leaf infiltration with 1 μ M of the indicated elicitor or without peptide. Subsequently, leaves were infected with 10⁵ cfu ml⁻¹ *Pst. DC3000*, and bacterial growth was assessed 1 and 2 days after infection. **(I)** Induction of *PROSCOOP12* in the same tissue and approach as described in B. The bars represent the mean of 6 biological replicates. Error bars show \pm SE of the mean. Significant differences according to Student's t-test results: *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Small endogenous peptides are known to be active at a very low concentration. In order to confirm this property for the SCOOP12 peptide we performed dose effect experiment by using the seedling growth experiment. The results show that flg22 causes an inhibition of seedling growth at concentrations as low as 1nM. A concentration of 100nM SCOOP12 causes a highly significant reduction in seedling fresh weight while the root length is less severely affected. Moreover, SCOOP12 inhibits seedlings growth with a concentration of 50nM. (Figure 11).

The SCOOP12 peptide regulates defense responses and root development

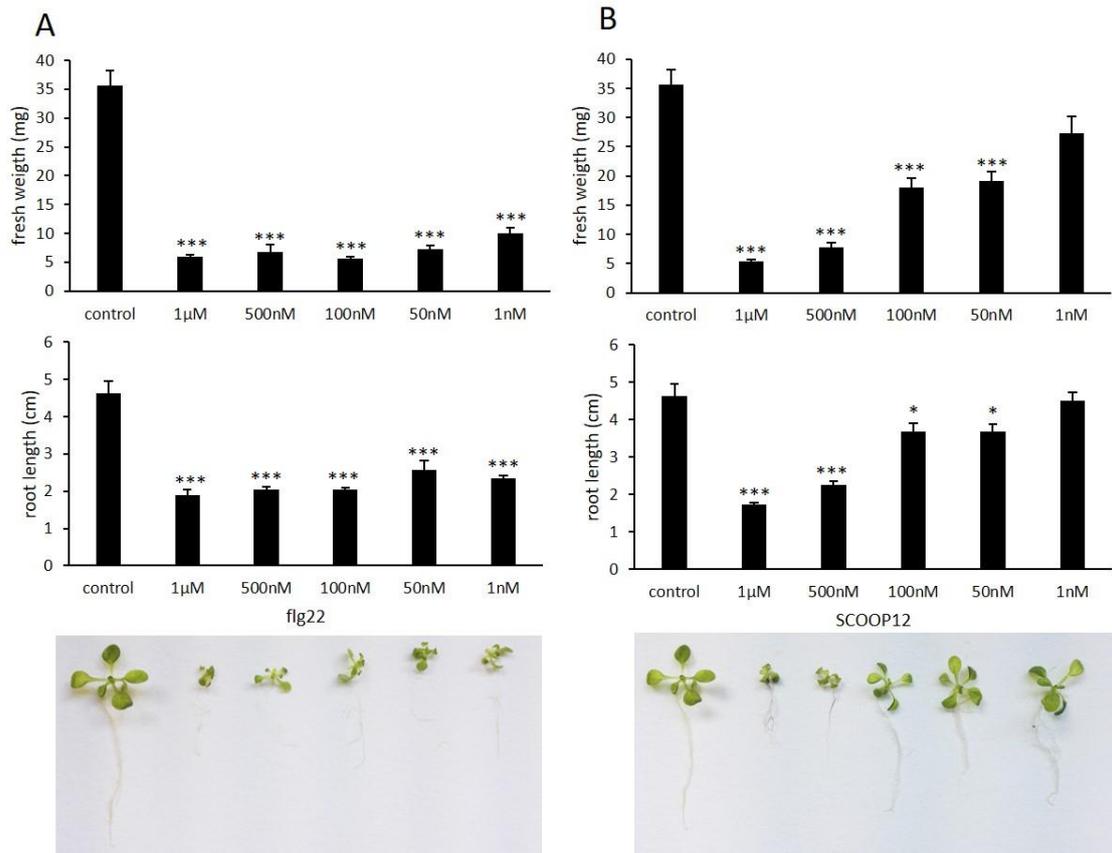


Figure 11: **Dose-dependent effect of SCOOP12.** Quantification of seedling growth inhibition. 5 days old seedlings were transferred from solid MS medium to liquid medium supplied with flg22 or SCOOP12 with the indicated concentration and were grown for additional 8 days before fresh weight and root length was quantified and pictures were taken. The bars represent the mean of 6 replicates. Error bars show \pm SE of the mean. Significant differences according to Student's t-test results: *, $P < 0.05$; ***, $P < 0.001$.

4.3.6. Pre-treatment with the SCOOP12 peptide protects Arabidopsis against Pseudomonas infection

It has previously been shown that priming of plants with the flg22 elicitor as well as with oligogalacturonides could result in enhanced tolerance against subsequent bacterial infections. For instance, plants pre-treated with these elicitors showed significantly reduced lesion size following an infection with *Botrytis cinerea* (Raacke et al., 2006; Ferrari et al., 2007). Using a similar assay, we found that plants pre-treated with flg22 as well as with SCOOP12 and AtPep1 were less susceptible to *Pseudomonas syringae* pv. *tomato* DC3000 infection (Figure 10H). The effect of the two endogenous peptides SCOOP12 and AtPep1 was weaker

than flg22, which is consistent with the fact that flg22 induced stronger defense response compared to SCOOP12 (Figure **10 AC**).

4.3.7. SCOOP12 induce the expression of PROSCOOP12 and PROPEP2

It has previously been shown that small endogenous peptides can induce the expression of their own precursors resulting in a positive feed-back loop. For instance, expression of several *PROPEP* genes can be induced by different *AtPep* peptides (Huffaker and Ryan, 2007). This led us to investigate the change in steady state transcript level of *PROSCOOP12* after SCOOP12 exposure. Moreover, we decided to add *AtPep1* in our assay for comparison since it is also known to induce transcription of another peptide precursor, *prePIP1* (Hou et al., 2014). The results show, that *PROSCOOP12* is upregulated by SCOOP12 in comparison to the control treatment (Figure **10 I**). Therefore, there is a positive feedback loop linking SCOOP12 to its putative precursor *PROSCOOP12*. Next, we wanted to know if SCOOP12 is capable of inducing the expression of *PROPEP* family members. However, SCOOP12 does not induce expression of *PROPEP1*, while *PROPEP2* is induced by a SCOOP12 application (Figure **12 AB**). Indicating a cross regulation between the two peptide families.

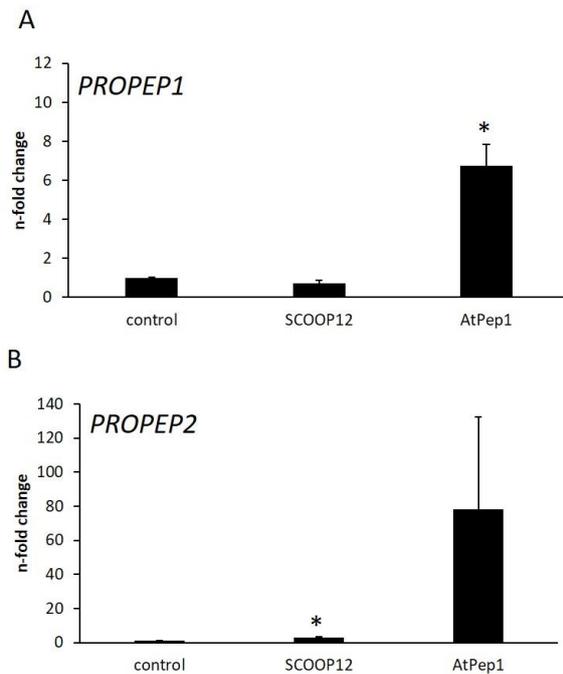


Figure 12: **Transcriptional response of *PROPEP1* and *PROPEP2* to SCOOP12 and AtPep1.** A) transcription of *PROPEP1* is induced by AtPep1 but not by SCOOP12. B) Transcription of *PROPEP2* increases 3 folds by SCOOP12 application. Expression levels were determined by normalization to *ACR12* transcripts, and bars indicate the fold change of transcription relative to the control treatment of two independent biological replicates. Error bars show the relative ± 1 SE of the mean. Significant differences according to Student's t-test results: *, $P < 0.05$.

4.3.8. *proscop12* does not show an altered response to *Pseudomonas* infection

It was shown that the overexpression of the AtPep1 precursor *PROPEP1* results in an enhanced resistance to the root pathogen *Pythium irregulare* (Huffaker et al., 2006). Moreover it was shown that the two precursors *PROPEP2* and *PROPEP3* are enhanced expressed by a *Pseudomonas* infection (Ross et al., 2014). However, it was so far not shown that a mutant of a *PROPEP* member is more susceptible to *Pseudomonas* infection. We decided to infect the *proscop12* plants in the *Ws* background with *Pseudomonas* (Figure 13). Even though we have found that the SCOOP12 peptide induces various defense responses, a knock-out in *PROSCOOP12* does not result in an altered response to *Pseudomonas* (Figure 13 AB).

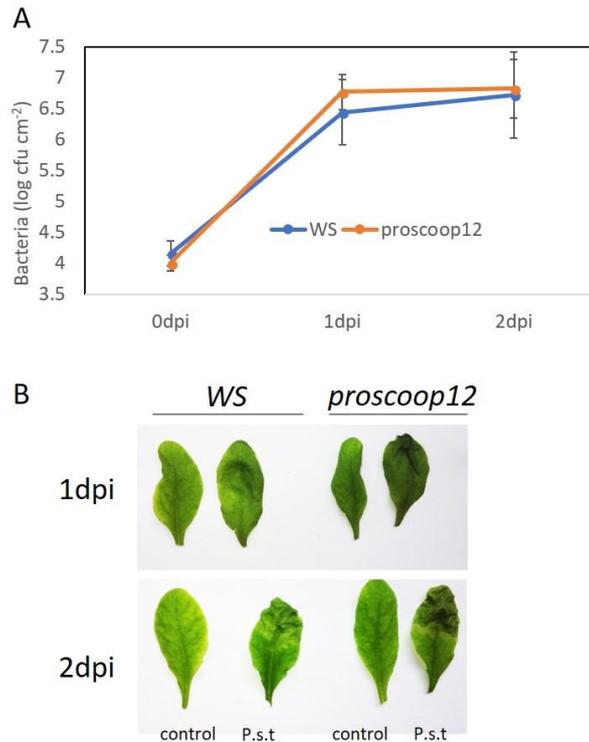


Figure 13: *P. syringae* pv *Tomato* DC3000 (*Pst* DC3000) (*P.s.t*) infection Assay of Wild-Type *Arabidopsis* (*WS*) and *proscoop12*. A) Leaves were infected with 10^5 cfu ml⁻¹ *Pst. DC3000*, and bacterial growth was assessed 1 and 2 days after infection. B) No phenotypic infection symptoms difference could be observed between wildtype and *proscoop12* plants.

4.3.9. SCOOP12 activity depends on the correct amino-acid order

In order to demonstrate the specificity of SCOOP12 sequence, we synthesized a peptide based on a randomized version of the same 13 amino acids and tested plant responses to this scrambled SCOOP12 (scSCOOP12). Furthermore, we synthesized peptides with double alanine replacements (SCOOP12 S5/7A) and single replacements (SCOOP12 S5A; SCOOP12 S7A) to test the importance of the two highly conserved serine residues on positions 5 and 7 of SCOOP12 (Figure 6B) for its activity. Plants treated with scSCOOP12 as well as with the modified peptides did not show seedling growth inhibition. Total seedling fresh weight as well as root length were not different from that of control plants (Figure 14). Finally, treatments with scSCOOP12, SCOOP12 S5/7A and SCOOP12 S5A did not induce a ROS burst and only SCOOP12 S7A resulted in a low, still significant ROS burst (Figure 14). These results highlight the importance of the amino acid order and the presence of the highly conserved serine residues for the perception of SCOOP12 by the plant.

The SCOOP12 peptide regulates defense responses and root development

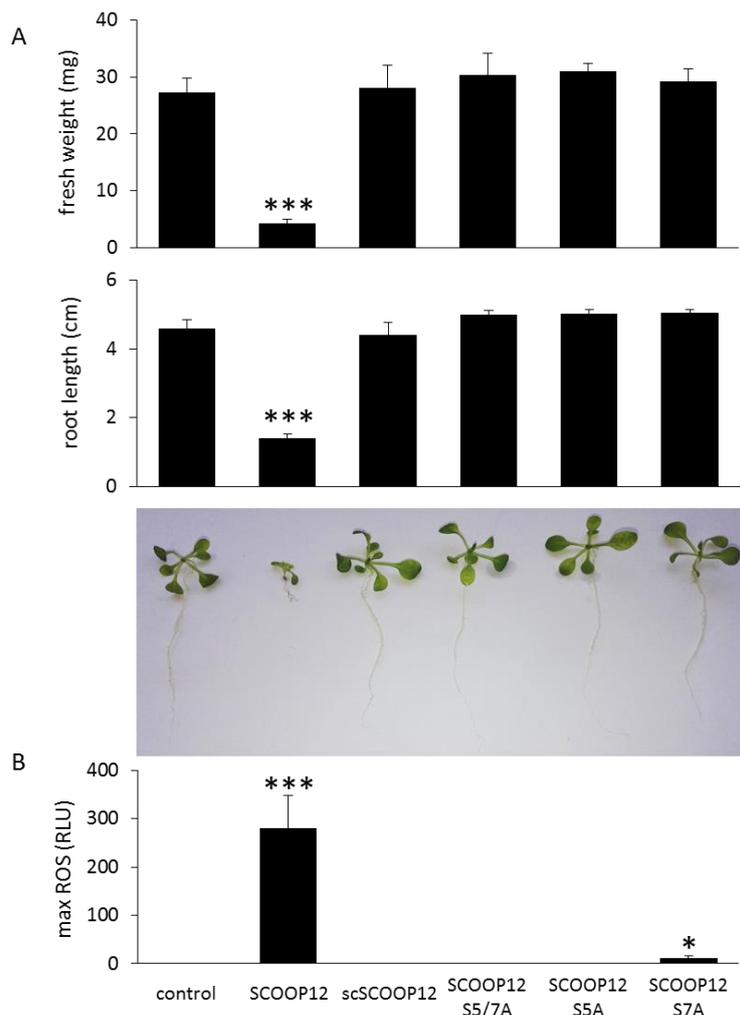


Figure 14: **SCOOP12 activity depends on the correct amino-acid order and two highly-conserved Serine.** Assays were carried out with scrambled peptide (scSCOOP12) and alanine replacements of conserved serine residues at position 5 and 7 of SCOOP12 (PVRSSQSSQAGGR) (SCOOP12 S5/7A; SCOOP12 S5A; SCOOP12 S7A). **(A)** Quantification of seedling growth inhibition with the indicated elicitors. Bars of quantified fresh weight and root length represent mean of six replicates. **(B)** Reactive oxygen species (ROS) in RLU (relative light units) production in wild-type Arabidopsis leaf-discs (Col-0), treated with $1\mu\text{M}$ for each peptide or without elicitor (control). Graphs display averages of 12 replicates. Error bars show $\pm\text{SE}$ of the mean. Significant differences according to Student's t-test results: *, $P < 0.05$; ***, $P < 0.001$.

4.3.10. SCOOP12 is only perceived by Arabidopsis and Brassica napus

Next, we wanted to test the conservation of plant responses to SCOOP12. For that purpose, plants were selected in which we identified *PROSCOOP* homologues (*Brassica napus*, Figure 7) and plants for which this gene family has not been identified (*Nicotiana benthamiana* and *Lycopersicon esculentum*). We measured ROS production following application of SCOOP12

in these plants and included flg22 as a positive control. We detected a ROS burst caused by flg22 in all four plant species. On the other hand, SCOOP12 only resulted in a ROS burst in *A. thaliana* and at a lower, yet still significant level, in *B. napus* (Figure 15). Therefore, only the two plant species containing homologues of the PROSCOOP gene-family members, showed a response to SCOOP12 treatments.

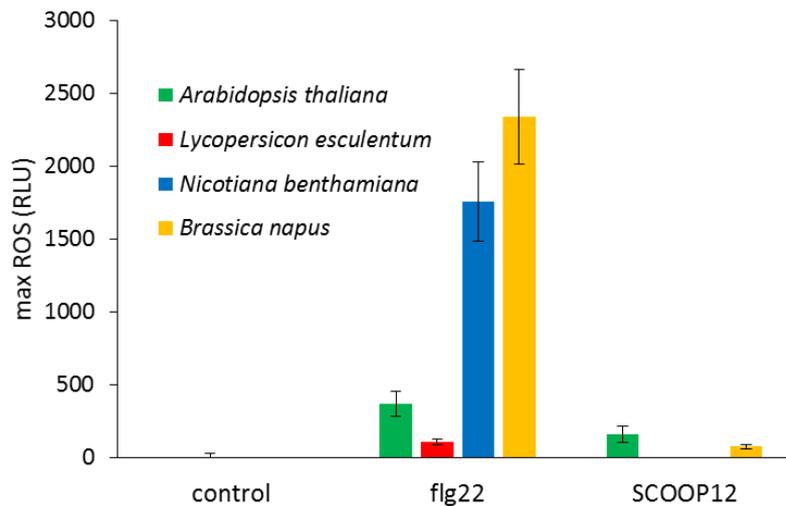


Figure 15: **ROS burst measurements on selected plant species treated with SCOOP12.** ROS burst assay was performed on *Arabidopsis thaliana* (Col-0), *Lycopersicon esculentum*, *Nicotiana benthamiana* and *Brassica napus*. The flg22 and SCOOP12 peptides were added to a final concentration of 1 μ M. Bars display the average of the maximum ROS burst in RLU (relative light units) of 12 replicates.

4.3.11. The BAK1 co-receptor is involved in SCOOP12 perception

A well characterized co-receptor of several receptors of small peptides is BRI1-associated kinase1 (BAK1). It has been shown to dimerize with BRI1 (Brassinosteroid-Insensitive 1), the brassinosteroid receptor (Li et al., 2002). Interaction of BAK1 with receptor like kinases that act as elicitor receptors, was proposed to be due to conformational changes occurring after ligand binding which results in the formation of the receptor complex (Chinchilla et al., 2009; Liu et al., 2017). To test if BAK1 is involved in the perception of SCOOP12, a seedling growth inhibition assay was performed on *bak1-4* plants. Compared to wild-type controls, *bak1-4* plants did not display any significant growth inhibition upon SCOOP12 treatment (Figure 16A). In order to identify the SCOOP12 receptor, the same approach was carried out on *fls2* (the

flg22 receptor) and *pepr1/pepr2* plants. Contrary to BAK1, our results suggest that these receptors are not involved in the perception of SCOOP12 (Figures 16BC).

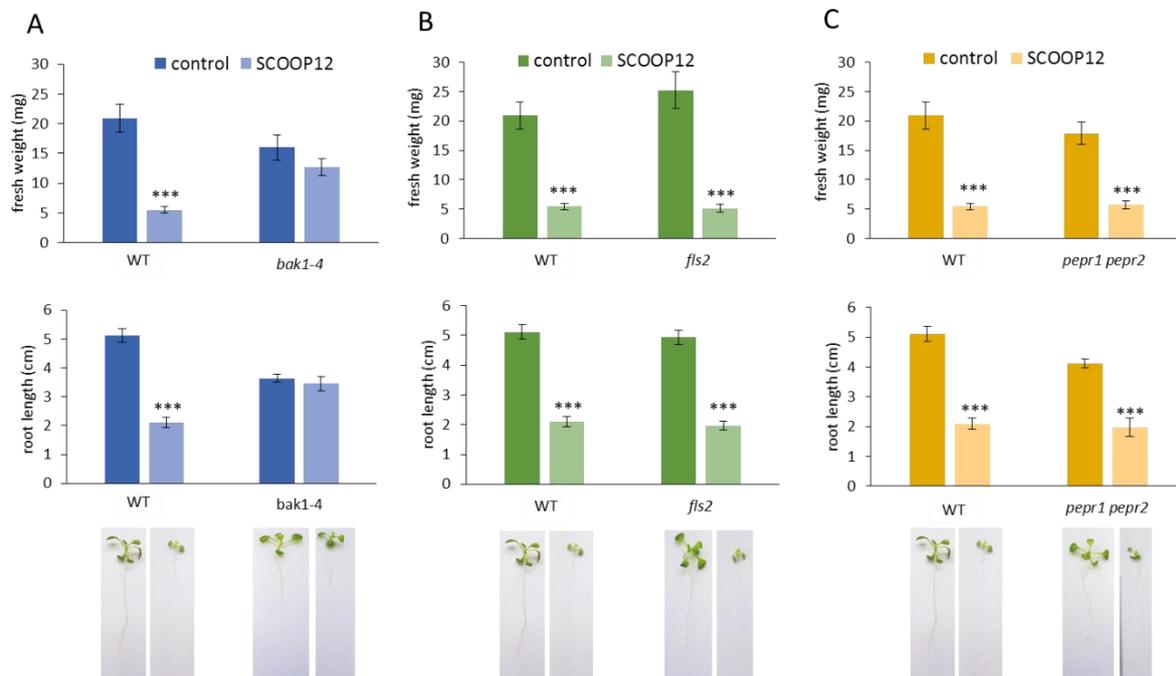


Figure 16: **Seedling growth inhibition assay on selected receptor mutant backgrounds. (A)** *bak1* plants were insensitive to SCOOP12. Neither fresh weight (top) nor root length (center) were affected by SCOOP12 treatment. The FLS2 **(B)** and PEPR1/PEPR2 **(C)** receptor mutants were not affected in their perception of SCOOP12. Plants were grown for 8 days in presence of 1 μ M SCOOP12 or control solution. Bars of quantified fresh weight and root length represent mean of six replicates. Error bars show \pm SE of the mean. Significant differences according to Student's t-test results ***, $P < 0.001$.

In order to confirm the involvement of BAK1 in the perception of SCOOP12 we tested a double mutant *bak1-5 bkk1-1*. The mutant *bak1-5* harbors a single amino acid substitution and does, in contrast to *bak1-4*, still accumulate wild type level of BAK1-5 protein (Roux et al., 2011). However, the double mutant *bak1-5 bkk1-1* is nearly completely impaired in defense responses caused by flg22, elf18 and AtPep1 (Roux et al., 2011). The results show that *bak1-5 bkk1-1* is nearly completely impaired in the perception of SCOOP12. However, the root length of *bak1-5 bkk1-1* treated with SCOOP12 is significantly shorter compared to the untreated control (Figure 17).

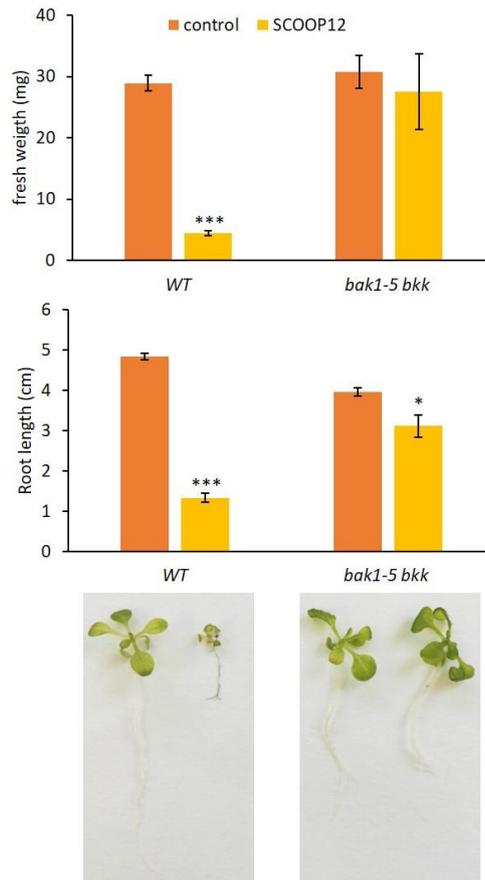


Figure 17: **Seedling growth inhibition assay using the *bak1-5 bkk1-1* double mutant.** *bak1-5 bkk1-1* plants are nearly completely impaired in SCOOP12 perception. Fresh weight is not affected by SCOOP12 treatment while root length shows a reduction upon SCOOP12 treatment. Plants were grown for 8 days in presence of 1 μ M SCOOP12 or control solution. Bars of quantified fresh weight and root length represent mean of six replicates. Error bars show \pm SE of the mean. Significant differences according to Student's t-test results ***, $P < 0.001$; *, $P < 0,05$.

4.3.12. SCOOP12 rapidly activates phospholipid signaling pathways in Arabidopsis cell suspensions

Lipid signaling pathways act as multifunctional regulatory mechanisms in plants. They incorporate several groups of inducible enzymes that convert membrane phospholipids into signaling molecules. Phosphatidic acid (PA) is a well-known biologically active lipid that is produced in response to numerous hormonal and stress signals including, notably, flg22 (van der Luit et al., 2000). Here, we show (experiments done by collaborators at the iEES in Paris) that application of SCOOP12 induces an accumulation of PA in Arabidopsis cell suspensions (Figure 18). This effect is observed as early as 5 min following SCOOP12 application in a low concentration of 100nM (Figure 18 BC). The scSCOOP12 had no effect on PA accumulation.

Two modes of PA accumulation are known: PLD-dependent via direct hydrolysis of membrane phospholipids and DGK-dependent via phosphorylation of diacylglycerol (DAG). In our experiment a labelling protocol that favors visualization of DGK-derived PA was used (Ariz and Munnik, 2013). PIP₂ is a substrate to phosphatidylinositol-specific phospholipase C (PI-PLC) that produce DAG. We have also observed that the level of phosphatidylinositol 4,5-bisphosphate (PIP₂) is transiently reduced following SCOOP12 treatment (Figure 18 B). These results suggest that SCOOP12 initiates a signaling cascade implicating PI-PLC (causing the depletion of PIP₂) and subsequent production of PA via phosphorylation of DAG by DGK.

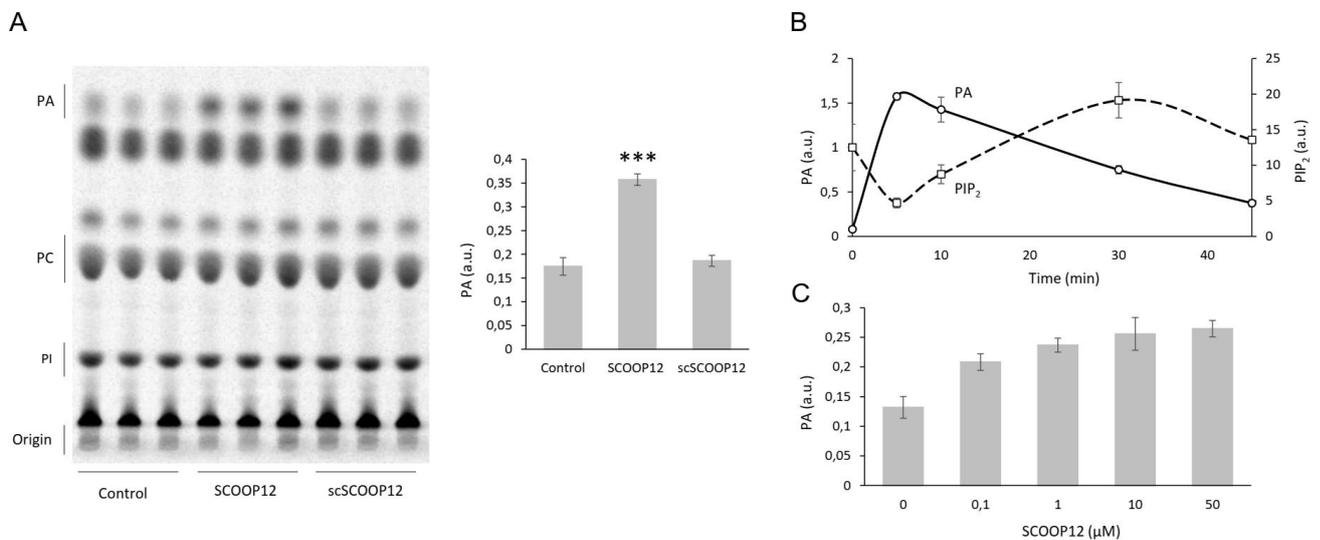


Figure 18: Rapid activation of PA production in Arabidopsis cell suspensions following treatment with SCOOP12. (A) Separation of P³³-labelled lipids using thin-layer-chromatography with contrasting effects of SCOOP12 (10μM) and scrambled scSCOOP12 (10μM) on the level of PA accumulation visible after 5 min of treatment. Significant differences according to Student's t-test results: ***, P < 0.001. (B) Time-scale of the SCOOP12 (1μM) influence on PA and PIP₂ accumulation in Arabidopsis cell suspensions (C) Dose-scale of the SCOOP12 influence on PA and PIP₂ accumulation in Arabidopsis cell suspensions after 5 min of treatment. All experiments were performed with at least three biological replicates. Error bars show ±SE of the mean. PA, phosphatidic acid; PIP₂, phosphatidylinositol 4,5-bisphosphate; PI, phosphatidylinositol; PC, phosphatidylcholine; a.u., arbitrary units.

4.4. Discussion

The comparison of the PROSCOOP family with other previously published genes encoding such secreted peptides highlights numerous shared features but also interesting specificities.

The SCOOP12 peptide regulates defense responses and root development

At the structural level, the PROSCOOP proteins distinguish themselves by the absence of a highly conserved C-terminal region. Indeed, the detected motives are quite divergent compared to the other PTMP precursors (Matsubayashi, 2011). This divergence could explain the fact that no PROSCOOP homologs could be detected outside of the *Brassicaceae* genomes. This restricted phylogenetic profile is opposite to the other described secreted peptides which are conserved both in monocot and eudicot plants. Furthermore, contrary to the majority of the known PTMPs, the conserved motifs are not localized at the C-terminal extremity of their precursors, and their maturation could involve two steps of proteolytic processing or a trimming step (Matsubayashi, 2011).

Out of the 14 Arabidopsis PROSCOOP proteins, three include two duplicated SCOOP motifs (Figure 6), reminiscent of the previously described cases of the CEP and PIP families (Roberts et al., 2013; Vie et al., 2015) and also of the CLE18 protein in which each copy of the conserved CLE motifs has a specific function (Murphy et al., 2012). The motif composition classifies SCOOP in the superfamily of 'SGP-rich peptide' among PIP, CLE, IDA, PEP and CEP families (Hou et al., 2014). At the functional level, the triggering of ROS burst, *FRK1* transcription and callose deposition move SCOOP12 close to the cytosolic *AtPep* and apoplastic PIP families (Huffaker et al., 2006). Our results suggest a functional link between *AtPep1* and SCOOP12 since both peptides induce the transcription of *PROSCOOP12* (Figure 10 I). This collaboration between different peptide families has also been described with *AtPEP1* and PIP1 which act cooperatively to amplify triggered immunity. Furthermore, the signaling induced by *AtPep1* (Schulze et al., 2010), PIP1 (Hou et al., 2014) and SCOOP12 (Figure 16, 17) is dependent on the BAK1 co-receptor. Our results also show that *proscop12* in WS background does not show an altered resistance to *Pseudomonas syringae* infection. Similar results were obtained for the *AtPep* receptor mutants (*pepr1* and *pepr2*). These mutants were not more resistant, while a pre-treatment of *AtPep1-6* peptides increased the resistance to a subsequent *Pseudomonas* infection (Yamaguchi et al., 2010).

In addition to the role of peptides as amplifiers of the immune response, these peptides are involved in root development but via different mechanisms. A high number of PTMP are expressed in the root tip and play a role in primary root development. One of the most prominent one is the CLE family, which is involved in developmental processes in the root apical meristem (Casamitjana-Martínez et al., 2003; Stahl and Simon, 2009; Murphy et al., 2012). The *cle40* mutant, one member of this family, shows an enlarged root phenotype. This

is due to repression of *WUSCHUL* (*WUS*) expression (Hobe et al., 2003; Stahl et al., 2009). It is possible that also *PROSCOOP12* influences the expression of important root growth factors. In figure **12** we show that transcription of *PROPEP2* is induced by SCOOP12 application. Previously it was reported that an overexpression of *PROPEP1* and *PROPEP2* lead to an increased root and aerial growth (Huffaker et al., 2006). The overexpression of the PIP1 precursor or its exogenous application inhibits Arabidopsis root growth as described for CEP (Roberts et al., 2013), AtPep1 (Poncini et al., 2017) and SCOOP12 peptide (Figure **10F**). Acting as growth factors and contrary to SCOOP12, the PTMP PSK and PSY1 are involved in root elongation (Amano et al., 2007; Matsuzaki et al., 2010). Our results could be a first indication of a complex root growth regulation system implemented by the interaction of multiple peptide families.

However, these comparisons show that despite common structural and functional characteristics, the SCOOP family is different from previously described secreted peptides. The divergence observed in the C-terminal sequence of PROSCOOP proteins suggest a large set of biological functions through a diversity of receptors which will be the targets of future studies. The functions of such plant secreted peptides at the boundaries of development- and stress-signaling pathways open the way to future strategies that jointly consider product quality/quantity and new resistance traits.

In conclusion, SCOOP12 belongs to a new family of putatively secreted peptides (specific to the Brassicaceae species) displaying all the structural features of post-translationally modified peptides. SCOOP12 could play a role in the moderation of defense responses, as well as root growth.

4.5. Material and methods

4.5.1. Plant material

Plant material used was wild-type *Arabidopsis thaliana* L. Heynh cultivar 6 Columbia (Col-0) as well as the cultivar Wassilewskija (Ws) and the mutants *proscop12* (T-DNA line FLAG_394H10 in Ws background, primers used for genotyping are detailed in Table **1**). The *proscop12* mutant in Col-0 background was created using the CRISPR-Cas9 approach. We searched *proscop12* gene-specific sgRNA and potential off-target sites in the Arabidopsis

The SCOOP12 peptide regulates defense responses and root development

genome using the crispor tefor program (<http://crispor.tefor.net>). *bak1-4* (T-DNA line SALK_116202), *fls2* (Gomez-Gomez and Boller, 2000) and *pepr1/pepr2* described by (Flury et al., 2013). All *in vitro* plants (on Murashige and Skoog) were grown under short day conditions (photoperiod of 8h light at 22°C/16h dark at 21°C, with 70% of relative humidity). Plants used for all other assays were grown under long day conditions (photoperiod of 16h light at 22°C/8h dark at 21°C, with 60% relative humidity). *B. napus* (Darmor-bzh) and *L. esculentum* (Sweet Baby) were grown under short day conditions.

4.5.2. Root length experiment

Proscoop12 and *Ws* as well as *proscop12* and Col-0 seedlings were grown on solid MS plates for 2 weeks. Plates were placed in a vertical position. After 11 days, the primary roots length was measured and compared. For each genotype, two repetitions have been done with at least 10 seeds per plate.

4.5.3. Plant inoculation with *pseudomonas syringae* pv *Tomato DC3000* (*Pst DC3000*)

Wassilewskija and *proscop12* mutant plants were grown 5 weeks on soil. Eight leaves per plant were infiltrated with bacterial suspensions of at a concentration of 10^5 colony forming units (cfu/ml) (OD600 of 0.02) in sterile 10mM MgCl (also used for mock-inoculation) using a needleless syringe. Plants were maintained at high humidity. Samples were taken using a cork-borer (d=8mm) to cut one leaf discs per infected leaf. Leaf discs were ground in 10mM MgCl, diluted to the indicated concentration and plated as droplets of 10 μ l on YEB plates with the appropriate selection. Plates were incubated at 28°C and colonies counted two hours after the infection (0 dpi) as well as 1 and 2 dpi. Pictures were taken after 1 and 2 dpi.

4.5.4. Protection assay

Mature leaves of *Arabidopsis thaliana* plants were infiltrated by needleless syringe infiltration with the indicated elicitor peptide or control solution and were kept under long day growth conditions for 24h. The *P. syringae* pv *tomato DC3000* (*Pto. DC3000*) strain was grown in overnight culture on YEB medium plates supplemented with appropriate antibiotics. Cells were harvested from the plate and re-suspended in sterile 10mM MgCl and diluted to an OD600 of 0.02. Bacteria solution was needles syringe infiltrated into the pre-treated leaves.

The SCOOP12 peptide regulates defense responses and root development

Plants were maintained at high humidity. Samples were taken using a cork-borer (d=8mm) to cut one leaf discs per infected leaf. Leaf discs were ground in 10mM MgCl₂, diluted to the indicated concentration and plated as droplets of 10µl on YEB plates with the appropriate selection. Plates were incubated at 28°C and colonies counted two hours after the infection (0 dpi) as well as 1 and 2 days post infection. Eight plants were infected for each pre-treatment and sampling time point. The experiment was performed two times with similar results.

4.5.5. Seedling growth inhibition assay

Seedlings were germinated on MS agar and grown for 5 days before transferring one seedling per well to 24 well plates containing 500 µl MS media or MS media supplied with the indicated elicitor peptide to a final concentration of 1 µM (six replicates per elicitor peptide treatment). Photos were taken, fresh weight and root length were measures after 8 additional days.

4.5.6. Elicitor peptides

Peptides of flg22 (QRLSTGSRINSAKDDAAGLQIA), *Arabidopsis thaliana* Plant Elicitor Peptide 1 (AtPep1) (ATKVKAKQRGKEKVSSGRPGQHN), SCOOP12 (PVRSSQSSQAGGR), scSCOOP12 (GRPRSASSGSVQQ), SCOOP12 S5/7A (PVRSAQASQAGGR), SCOOP12 S5A (PVRSAQSQAGGR) and SCOOP12 S7A (PVRSSQASQAGGR) were obtained from Eurogentec SA (Angers, France) and diluted in water to the final concentration used for the assays.

4.5.7. Measurement of reactive oxygen species

For ROS assays leaf discs of three weeks old soil grown plants, were placed into each well of a white 96-well plate (Thermo Scientific, Waltham, USA) in 0,1 ml of water and kept in the dark overnight. For elicitation and ROS detection, horseradish peroxidase and luminol were added to a final concentration of 10 µg ml⁻¹ and 100 µM, respectively. Luminescence was measured directly after addition of elicitor peptides in a FLUOstar OPTIMA plate reader (BMG LABTECH, Offenburg, Germany).

4.5.8. Callose deposition

Leaf discs were vacuum infiltrated for 10 min with the indicated elicitor solution and kept floated in elicitor or control solution for 24h. After leaf discs were fixed and destained in 1:3

acetic acid/ ethanol until leaf tissue was completely transparent. After washing the leaf discs in 150 mM K₂HPO₄ for 30 min, the plant material was stained for 2 h in 150 mM K₂HPO₄ and 0,01% aniline blue. Callose depositions were quantified with a Leica DM1000 microscope equipped with a Qimaging Micropublisher 3.3 RTV camera using a DAPI filter.

4.5.9. Determination of gene expression by qPCR

Detached leaves of three weeks old plants were collected and floated for two hours in water supplied with the indicated elicitor or control solution. After the treatment, material was frozen and ground in liquid nitrogen. RNA from 100 mg of tissue was extracted using the NucleoSpin RNA plant extraction kit (Macherey-Nagel Hoerd, France). The DNase treatment was performed according to the manufacturer's recommendations. Per PCR reaction, complementary DNA was synthesized from 10 ng of total RNA extract with oligo(dT) primers using Moloney Murine Leukemia Virus Reverse Transcriptase according to the manufacturer's instructions (Promega). For quantitative real-time reverse transcription PCR (qPCR) in a 96-well format, the Chromo4™ System (Bio Rad) was used. Expression was normalized to that of the gene *ACR12* (AT5G04740) using the qGene protocol (Muller et al., 2002). All the gene-specific primers used are detailed in Table 1.

Table 1: **List of Primer used in this study**

Name	Gene ID	Sequence fw	Sequence rev
<i>ACR12</i>	AT5G04740	TTGTTTCGATGATCGCCGGAA	TGGAACAACGTCGTCATCGT
<i>PROSCOOP1</i>	AT5G44565	AGCATCCTCTTTCACCATACCG	ATTCTGACCACCACCACCTC
<i>PROPEP1</i>	AT5G64900	TCTCCGACAACGTCCTCTCC	ACGGCCTGAGCTAACTTTCT
<i>PROPEP2</i>	AT5G64890	CGGTAACCTTTAACCAGCCGG	TTAGTTTGCCAGGACGACC
<i>FRK1</i>	AT2G19190	TAGATGCAGCGCAAGGACTA	ACCGCTTCCTTCAACAGAGA
primers used for genotyping of <i>proscop12</i>			
<i>ACT2</i>	AT3G18780	CTAAGCTCTCAAGATCAAAGGC	AACATTGCAAAGAGTTTCAAGG
<i>PROSCOOP12</i>	AT5G44585	ATGGGTCAAGTTCTAATTGTGC	TAATCTATGGCGATAGGATCAGC

5. The SCOOP family contains several defense-response inducing peptides that can regulate root growth

Parts of this chapter are published in modified version in Gully et al., (2018): “The SCOOP12 peptide regulates defense response and root development in *Arabidopsis thaliana*”. In this chapter my contribution to this paper included the complete experimental part (excepted the *in-silico* prediction of the applied peptides) and data compilation. This chapter could be the onset of a second publication and additional future work introducing additional peptides of the SCOOP family apart of SCOOP12 as well as interesting root phenotypes caused by members of the SCOOP peptide family.

5.1. Abstract

Small endogenous peptides are involved in controlling plant defense responses as well as most plant regulatory processes. Especially the regulation of root growth is under focus of research on small endogenous peptides. Small peptides have an important role in root development. Next to classical plant hormones Small peptides are a growing class of regulatory molecules. These molecules are involved in many aspects of root development. This includes the meristem maintenance, lateral root development, gravitropic response and vascular formation. Here we extend the knowledge of the recently discovered small peptide family SCOOP. Here, we show that the SCOOP family contains several defense responses inducing peptide in *Arabidopsis*. Additionally, we show an interesting effect of SCOOP peptides on root morphology and development. In conclusion we show that the SCOOP family is very complex and these peptides play an important role in root metabolisms or development.

5.2. Introduction

Plants are complex organisms and regulation of plant growth, development, reproduction and response to the various environmental stresses need to be tightly regulated by a complex network of signaling pathways. For a long time, it was considered that plant hormones like salicylic acid, ethylene, jasmonic acid, auxins, gibberellins, cytokinins, abscisic acid and brassinosteroids are the main signaling molecules during these processes. However, in recent years it was found that the plant regulation system is more complex than initially proposed. An increasing number of small peptides have been discovered and lead to an increased intricacy in nearly all facets of plant development and defense. The large number of small peptides can be classified into two different categories. The first contains primary endogenous danger signals which are passively released upon host damage and secondary endogenous peptides. The secondary endogenous peptides are actively processed and released upon tissue damage. Peptides of the second category can be divided into peptides which are processed and released upon an herbivore attack or a microbial infection and peptides which have been linked to the regulation of plant growth and development. These peptides are referred to as phytocytokines (Gust et al., 2017). This category contains peptides like CLAVATA3/EMBRYO-SURROUNDING REGION (CLE) peptides (Kondo et al., 2006), AtPeps (Yamaguchi and Huffaker, 2011), AtPIPs (Hou et al., 2014), RALFs (Murphy and De Smet, 2014), phytosulfokines (PSK) (Matsubayashi et al., 2006) and the SCOOP peptide family described in this thesis. Out of these examples PSK, AtPeps, AtPIPs, RALF and SCOOP have been shown to be involved in regulation of plant immune responses to herbivore or microbial attack (Mosher et al., 2013; Hou et al., 2014; Murphy and De Smet, 2014; Yasuda et al., 2017). The perception of members of these peptide families is dependent on the SERK (SOMATIC EMBRYOGENESIS RECEPTOR KINASE) family member BAK1 (Hou et al., 2014; Yasuda et al., 2017). BAK1 serves as coreceptor to various of these peptides. At a functional level phytocytokines have been shown to play key roles in root development. After proteolytic post-translational modification, whereas the peptide is processed from a precursor protein, it is secreted out of the cell and regulates many aspects of root growth and development including gravitropism, meristem maintenance, lateral root development and protoxylem differentiation (Delay et al., 2013). The root growth is also strongly influenced by stresses.

The SCOOP family contains several defense inducing peptides

Plants can redirect root growth and morphology to diminish stress exposure. This plant response is referred to as stress-induced morphogenic response. These responses can be summarized by three components. The elongation of cells can be inhibited, cell division can be locally stimulated, and cell differentiation can be altered (Potters et al., 2007). Small peptide families contain a different number of members. The CLE is a large family that contains 32 peptides which are expressed in various tissues (Jun et al., 2010). The *AtPIP* family contains at least 11 members (Hou et al., 2014) and the *AtPep* family at least 8 members (Bartels and Boller, 2015). This shows that peptide families can contain various numbers of small peptides, whereas members may play different roles in plant development and defense. For certain families (*AtPep* and *PIP*) it was shown that the small processed peptide can induce the expression of its own precursor protein, effectively resulting in a positive feedback loop. Expression of different *PROPEP* proteins have been shown to be induced by various processed peptides (Huffaker and Ryan, 2007). Moreover, expression of *prePIP1* is induced by treatments with the *AtPIP1* small peptide (Hou et al., 2014). In the previous chapters, we show that the recently discovered *SCOOP* peptide family, includes various defense response activating peptides. In this chapter we report on the roles of three *SCOOP* peptides on root development and root stress responses.

5.3. Results

5.3.1. SCOOP12 and AtPep1 induce the expression of various PROSCOOP family members

It has previously been shown, that small endogenous peptides can induce the expression of their own precursors resulting in a positive feed-back loop. For instance, expression of several *PROPEP* genes can be induced by different *AtPep* peptides (Huffaker and Ryan, 2007). This led us to investigate the change in steady state transcript level of all 14 *PROSCOOP* family members after *SCOOP12* exposure. Moreover, we decided to add *AtPep1* in our assay for comparison since it is also known to induce the transcription of another peptide precursor, *prePIP1* (Hou et al., 2014). The results show that *PROSCOOP 2, 7, 8, 12* and *13* are upregulated by the *AtPep1* treatment (Figure 19). Most importantly, the direct precursor *PROSCOOP12* is upregulated by *SCOOP12* in comparison to the control treatment (Figure 10,19). Therefore, there is a positive feedback loop linking *SCOOP12* to its precursor *PROSCOOP12* but also of

The SCOOP family contains several defense inducing peptides

other members of the *PROSCOOP* family such as *PROSCOOP7*. These results suggest that there is a feedback loop of SCOOP12 to its precursor and to *PROSCOOP7* and that *AtPep1* is capable of inducing five members of the *PROSCOOP* family.

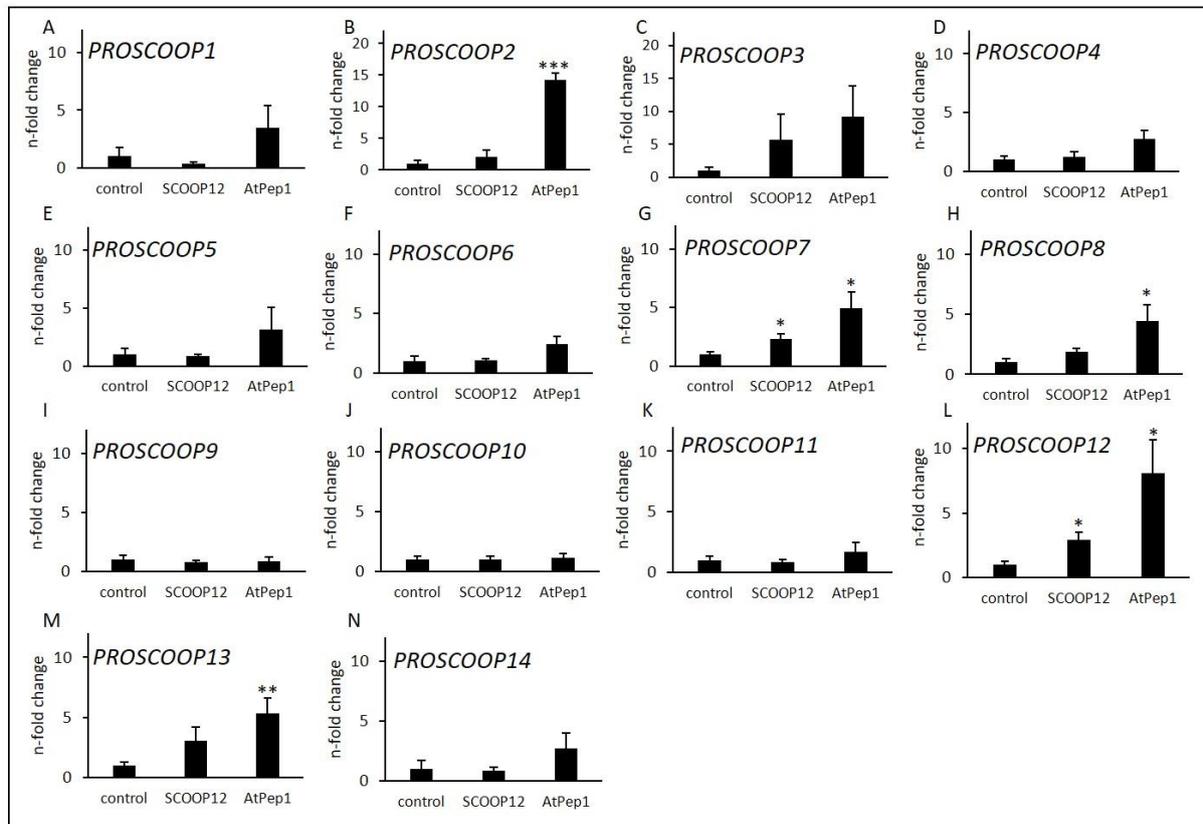


Figure 19: **Transcriptional response of the *PROSCOOP* gene family to SCOOP12 and *AtPep1*.** SCOOP12 and *AtPep1* induce the expression of several *PROSCOOP* gene family members. Expression level of the *PROSCOOP* gene family members (A to N) were determined by qPCR with a normalization to *ACR12* transcripts, bars indicate the fold change of transcription relative to the control treatment of at least five independent biological replicates. Error bars show the relative ± 1 SE of the mean. Significant differences according to Student's t-test results: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

5.3.2. Several SCOOP peptides induce ROS burst in Arabidopsis

In line with our previous results and the induction of *PROSCOOP* family member gene expression by SCOOP12 we decided to focus on the highly conserved motif found in all *PROSCOOP* family members (Figure 6B). We synthesized peptides covering the motif of all 14 family members (Table 2). The peptides were named according to their putative precursor

The SCOOP family contains several defense inducing peptides

protein. The motif contains two highly conserved serine (S) residues on position 5 and 7 of the peptides. Excepted of SCOOP3 all synthesized peptides harbor these two amino acids.

Table 2: **List of peptides, covering the identified motif on the corresponding PROSCOOP family member.** In total 14 PROSCOOP family members were identified. All 14 harbor the highly conserved motif. The peptides covering two highly conserved serine residues on position 5 and 7 of the peptides (marked in red). SCOOP3 is the only exception, here the serine on position 7 is replaced by a glutamate (marked in green) residue.

Peptide	Sequence
SCOOP1	ETP PSR SRR GGG G
SCOOP2	PVR SSR SPR SPS F
SCOOP3	ELR PSS EWR RKM I
SCOOP4	ASF HSA SPK DKG P
SCOOP5	IVR RSR SQR GRQ Y
SCOOP6	EAR PSK SKK GGG R
SCOOP7	RAG PSK SGQ GGG R
SCOOP8	DFE GS I SGQ AGG G
SCOOP9	GTG PSH SGH GGS S
SCOOP10	FTG PSG SGH GGG R
SCOOP11	DVG ASS SGQ GGG R
SCOOP12	PVR SSQ SSQ AGG R
SCOOP13	YLP PSK SRK GK G P
SCOOP14	FVP PST SHK GQG P

In this extensive screen we then wanted to test if apart of SCOOP12 also other peptides of this family have the potential to induce defense responses in Arabidopsis. Like previously described in chapter 4 we first tested short term defense and tested all SCOOP peptides for their ability to induce a ROS burst in Arabidopsis. The results show that indeed 9 peptides are capable to inducing a significant accumulation of ROS. The five peptides SCOOP3, 8, 10, 11 and 14 did not trigger a response. Notably, the response to flg22 was stronger in comparison to all of the active SCOOP peptides (Figure 20). The ROS induction by SCOOP12 is comparable to that of SCOOP6 and 7. Interestingly, SCOOP7 showed a response comparable to SCOOP12 in intensity. This is well in line with our finding that expression of PROSCOOP7, the putative precursor of SCOOP7 is induced by SCOOP12 (figure 19).

The SCOOP family contains several defense inducing peptides

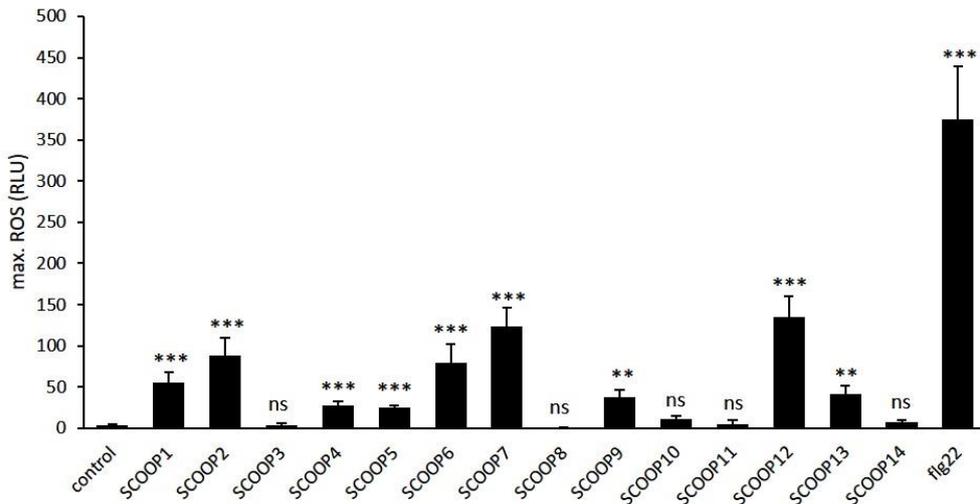


Figure 20: **ROS induction by the SCOOP peptide family.** Reactive oxygen species (ROS) in RLU (relative light units) production in wild-type Arabidopsis leaf-discs (Col-0), treated with 1 μ M for each peptide or without elicitor (control). Graphs display averages of 12 replicates. Error bars show \pm SE of the mean. Significant differences according to Student's t-test results: ns (not significant), **, $P < 0.01$; ***, $P < 0.001$.

5.3.3. The PROSCOOP family contains several growth inhibiting peptides

Next, we wanted to know if also long-term defense responses are induced by the SCOOP peptides. Therefore, we grew seedlings in media supplied with the 14 SCOOP peptides. A continuous activation of defense responses is known to inhibit the plants growth (Walters and Heil, 2007). Our results show that most SCOOP peptides induce an inhibition of Arabidopsis seedling growth. The peptides SCOOP3, 8 and 11 did not significantly inhibit seedling growth (Figure 21AB). In comparison with the obtained results of ROS accumulation we found that the peptides SCOOP10 and 14 do induce a seedling growth inhibition but no ROS accumulation (Figure 20,21). SCOOP10 induces a significant inhibition of root growth (Figure 21B) but the total fresh weight is not affected in comparison to the control treatment (Figure 21A). The repression of root growth and total fresh weight is comparable to that of control treatments with AtPep1 and flg22. The most pronounced inhibition of root growth was induced by SCOOP12, 6 and 7.

The SCOOP family contains several defense inducing peptides

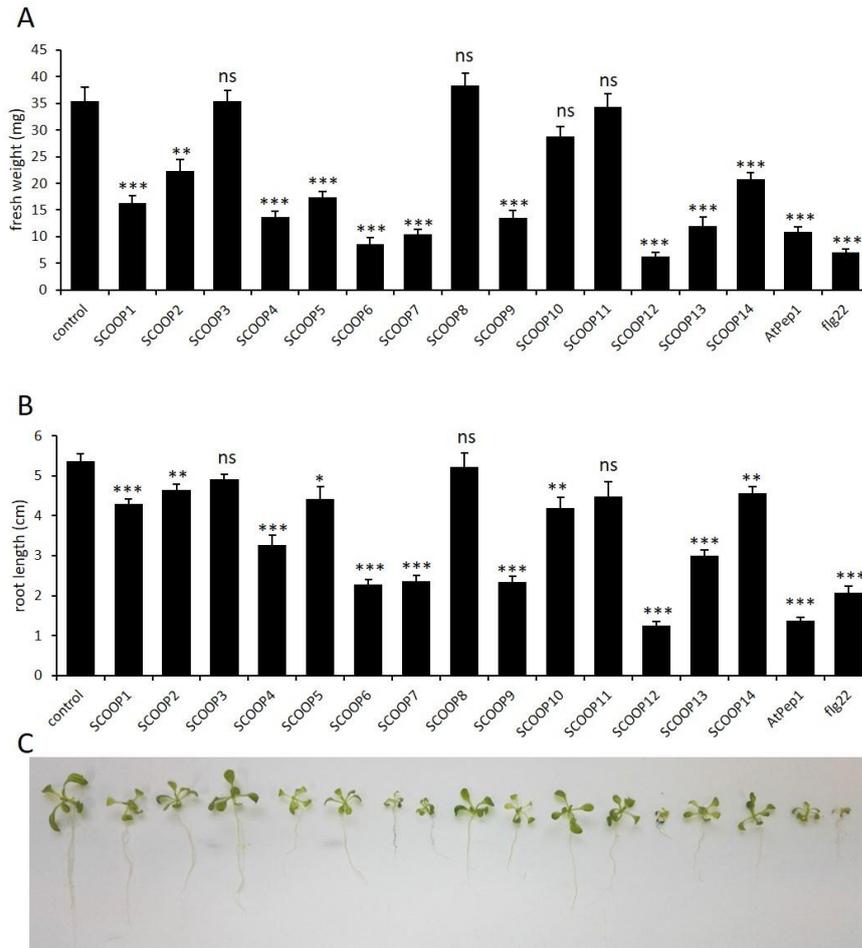


Figure 21: **Quantification of seedling growth inhibition.** 5 days old seedlings were transferred from solid MS medium to liquid medium supplied with the indicated elicitors (all applied in a final concentration of $1\mu\text{M}$) and were grown for additional 8 days before fresh weight and root length was quantified and pictures were taken. The bars represent the mean of 6 biological replicates. Error bars show $\pm\text{SE}$ of the mean. Significant differences according to Student's t-test results: ns (not significant) **, $P < 0.01$; ***, $P < 0.001$.

5.3.4. Exogenous application of SCOOP12 induces a strong root pigmentation

In chapter 4 we showed that *proscop12* displays an enhanced root growth. That was a reason for us to focus our observations on root tissue. During the seedling growth inhibition experiment (Figure 21) we noticed a strong root pigmentation caused by SCOOP12 but not by flg22 or AtPep1 (Figure 22A). The elicitors were added in a final concentration of $1\mu\text{M}$ to the growth media. We assumed that SCOOP12 causes a strong hypersensitive response in the roots and therefore cell death of affected cells. To investigate this hypothesis, we performed a cell death staining with neutral red. The staining solution stains exclusively intact and alive

The SCOOP family contains several defense inducing peptides

cells. The experiment showed that the roots are still intact, and no cell death is caused by the SCOOP12 treatment (Figure 22B).

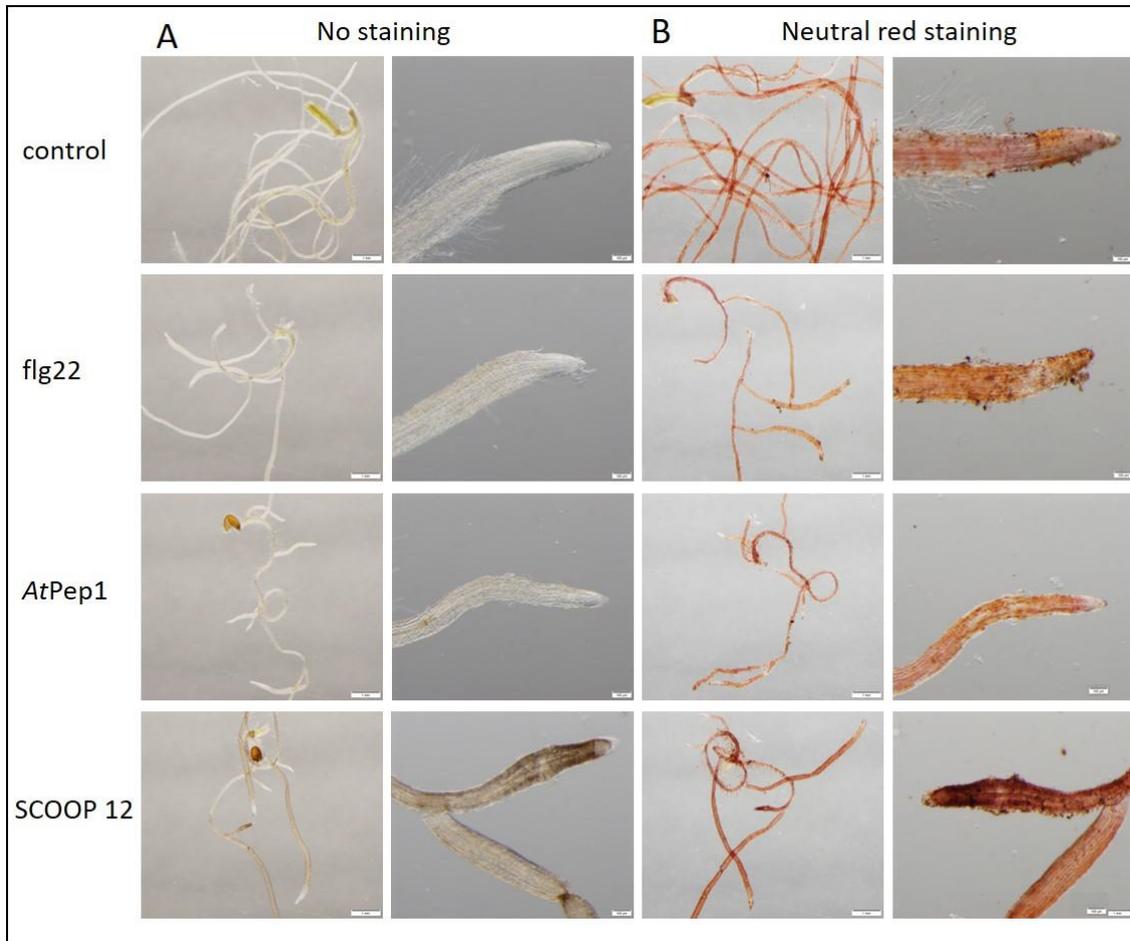


Figure 22: **SCOOP12 induces a strong root pigmentation.** Seedlings were treated as before described for the seedling growth inhibition assay. A) Roots of seedlings grown for 8 days in media supplied with the indicated elicitor peptide applied in a final concentration of 1 μ M. B) neutral red staining of the same roots shown in A.

5.3.5. Root pigmentation is caused by SCOOP6 and SCOOP7 in a tissue specific manner

In our previous results, we showed that the inductions of defense responses are most pronounced for the peptides SCOOP12, 6 and 7. These three peptides have the strongest effect on root growth. After the observation of a strong root pigmentation caused by SCOOP12, we decided to focus on SCOOP6 and 7 as other strong elicitor peptides. By the treatment of SCOOP12 we confirmed our previous observations. SCOOP12 causes a strong pigmentation of the entire root tissue. Moreover, the root architecture seemed to be altered. We could observe an increased number or an expansion of cells in the root transition and/or

The SCOOP family contains several defense inducing peptides

root elongation zone caused by SCOOP12 (Figure 22A). The roots treated with SCOOP12 have increased secondary root growth, while the primary root apical meristem show reduced growth (Figure 23). This altered root shape was not observed by treatments with SCOOP6 and 7. However, application of SCOOP6 causes a root pigmentation in a tissue specific manner. Exclusively the root differential and/or the elongation zone show root pigmentation with this peptide. The treatments with SCOOP7 lead to similar, but less pronounced, observations. These results are in line with our previous results on the effect of the SCOOP peptides on root growth, since these peptides show the strongest inhibition of root growth (Figure 21). Interestingly, the peptide SCOOP9 also had a similar effect on root growth yet no root pigmentation was observed (data not shown).



Figure 23: **Tissue specific root pigmentation caused by SCOOP6, 7, 12.** Seedlings were grown for 8 days in media supplied with the indicated elicitor peptide with a final concentration of 1 μ M or with control solution.

5.3.6. Perception of SCOOP6 and SCOOP7 is dependent on SERK family members

We have shown in chapter 4 that the perception of SCOOP12 depends on the SERK family member BAK1. In order to test if also SCOOP6 and SCOOP7 are perceived by members of the SERK family we decided to test the response of *bak1-5 bkk1-1* plants to this treatment. The *bak1-5* harbors a single amino acid substitution and does, in contrast to *bak1-4*, still accumulate the wild type level of BAK1-5 protein (Roux et al., 2011). However, the double mutant *bak1-5 bkk1-1* is almost completely impaired in defense responses caused by flg22,

The SCOOP family contains several defense inducing peptides

elf18 and AtPep1 (Roux et al., 2011). Therefore, we used this double mutant for treatments with SCOOP6 and SCOOP7. The results show that *bak1-5 bkk1-1* is almost completely insensitive to SCOOP6 (Figure 24AB), indicating the role of SERK family members as co-receptors of SCOOP family peptides perception. Moreover, the response to SCOOP7 is less pronounced than in the wildtype situation. However, this might be due to the higher variance within the replicates (Figure 24CD).

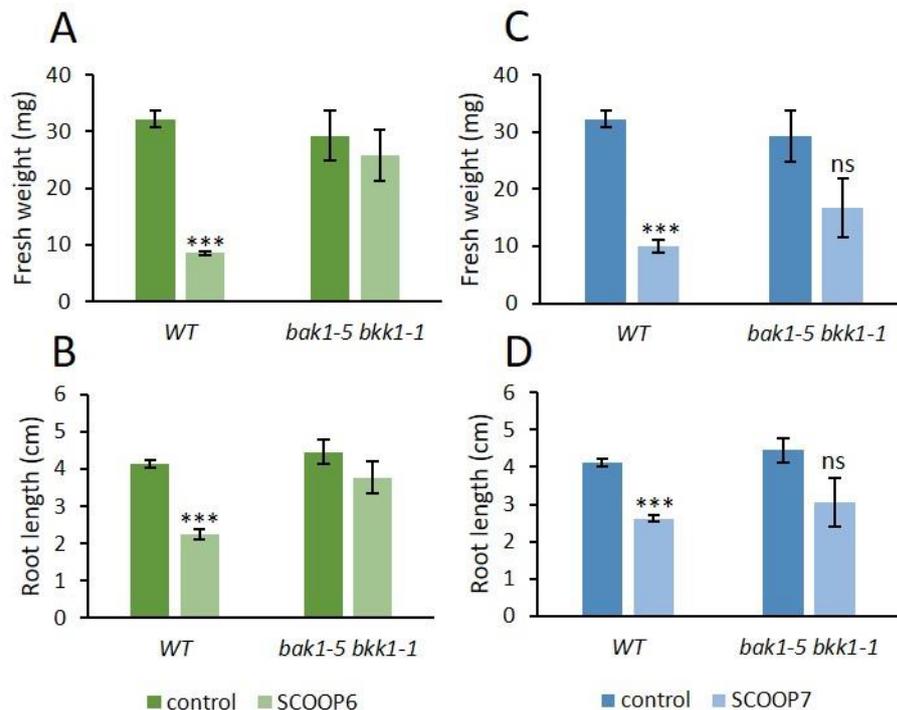


Figure 24: **Seedling growth inhibition assay with plants defective in SERK family members.** A) *bak1-5 bkk1-1* plants are insensitive to SCOOP6 and SCOOP7. Neither fresh weight A, C nor root length B, D were significantly affected by SCOOP12 treatment. Plants were grown for 8 days in presence of 1 μ M SCOOP12 or control solution. Bars of quantified fresh weight and root length represent mean of six replicates. Error bars show \pm SE of the mean. Significant differences according to Student's t-test results ***, $P < 0.001$.

5.4. Discussion

The SCOOP family harbors various peptides with interesting properties. In Table3 we summarized our current knowledge on the 14 members of the SCOOP family. It is important to note that all SCOOP peptides are based on a bioinformatic prediction approach, whereas

The SCOOP family contains several defense inducing peptides

all 14 PROSCOOP family members harbor a conserved motif. Out of the 14 synthesized peptides only 4 (SCOOP3, 8, 10, 11) do not show any activity in the applied assays. SCOOP14 did not induce a ROS burst but showed an inhibition of seedling growth. The strongest activity was observed for SCOOP12, as this peptide induced short- and long-term defense responses and a strong pigmentation of root tissue. The peptides SCOOP6 and SCOOP7 show similar results and an induction of a ROS burst, an inhibition of seedling growth as well as a pigmentation of roots. In chapter 3 we show that *bak1-4* is involved in the perception of SCOOP12. By testing the *bak1-5 bkk1-1* double mutant we confirmed that also SCOOP6 and SCOOP7 were perceived by members of the SERK family. However, by challenging a double mutant we cannot be sure which mutant is mainly responsible for the perception of SCOOP6 and 7. We intended to find a reason for the different activity of the SCOOP peptides. The activity of the SCOOP peptides seems to rely on the amino acid composition. By sorting the peptides by their activity and the charge of the single amino acids, we could not find a clear indication which amino acid is crucial for SCOOP peptide perception and activity (Figure 25).

Table 3: **Summary of our current knowledge on the SCOOP peptide family.** Experimental results are summarized from chapter 3 and 4. The peptide sequence and results of ROS, seedling growth inhibition, callose deposition assay are presented. As well as the involvement of tested SERK family members and the observed root pigmentation. Abbreviations: Yes, shows a significant or visible induction; no: no significant or visible induction; nd: not determined

Peptide	Sequence	ROS activity	Seedling growth inhibition	Root pigmentation	BAK1/BKK1 involved	callose deposition
SCOOP1	ETP PSR SRR GGG G	yes	yes	no	nd	nd
SCOOP2	FVR SSR SFR SPS F	yes	yes	no	nd	nd
SCOOP3	ELR PSS EWR RKM I	no	no	no	nd	nd
SCOOP4	ASF HSA SPK DKG P	yes	yes	no	nd	nd
SCOOP5	IVR RSR SQR GRQ Y	yes	yes	no	nd	nd
SCOOP6	EAR PSK SKK GGG R	yes	yes	yes medium	yes	nd
SCOOP7	RAG PSK SGQ GGG R	yes	yes	yes medium	yes	nd
SCOOP8	DFE GSI SGQ AGG G	no	no	no	nd	nd
SCOOP9	GTG PSH SGH GGS S	yes	yes	no	nd	nd
SCOOP10	FTG PSG SGH GGG R	no	no	no	nd	nd
SCOOP11	DVG ASS SGQ GGG R	no	no	no	nd	nd
SCOOP12	FVR SSQ SSQ AGG R	yes	yes	yes	yes	yes
SCOOP13	YLP PSK SRK GKG P	yes	yes	no	nd	nd
SCOOP14	FVP PST SHK GQG P	no	yes	no	nd	nd

However, the two highly conserved Serine residues on positions 5 and 7 of the 13 aa long peptides, seems to be important for the perception by the plant. SCOOP3 is the only peptide for which the polar Serine (S) residue is replaced with a negatively charged Glutamic acid (E) residue. SCOOP3 does not show any activity in the applied assays. Overall, it is notable that

The SCOOP family contains several defense inducing peptides

none of the inactive peptides (SCOOP3, 8, 10, 11) and weakly active peptide (SCOOP14) harbor positively charged aa at position 6 (Figure 25). Most of the active peptides (excepted of SCOOP4 and SCOOP12) have a positively charged aa at position 6. However, it remains unclear which effects the aa composition has on the peptide 3D structure since a reliable structure prediction is difficult for short peptides (Gupta et al., 2014). The SCOOP family contains at least 9 active peptides. The *AtPIP* family has at least 11 prePIP proteins whereas only 2 peptides are shown to have a defense inducing effect in *Arabidopsis* (Hou et al., 2014). The *AtPep* family contains at least eight PROPEP proteins and their corresponding small peptides. Out of the eight *AtPep* peptides the small peptides *AtPep 1* and *AtPep2* show the strongest defense inducing activity. The alignment of the 8 PROPEP proteins shows that the conserved motif is less variable and longer than it is the case for the SCOOP peptide family (Bartels et al., 2013). Indicating that the variability within the peptides and the short length of the conserved region might prevent a better prediction of members of peptide families. The synthesized peptides are based on the conserved motif found in all *PROSCOOP* genes. However, the *in planta* peptides are for the moment not known. The experimental determination and confirmation of all peptides could improve our knowledge about their properties and functional roles in plants.

Name	Peptide sequence												
SCOOP1	E	T	P	P	S	R	S	R	R	G	G	G	G
SCOOP2	P	V	R	S	S	R	S	P	R	S	P	S	F
SCOOP4	A	S	F	H	S	A	S	P	K	D	K	G	P
SCOOP5	I	V	R	R	S	R	S	Q	R	G	R	Q	Y
SCOOP6	E	A	R	P	S	K	S	K	K	G	G	G	R
SCOOP7	R	A	G	P	S	K	S	G	Q	G	G	G	R
SCOOP9	G	T	G	P	S	H	S	G	H	G	G	S	S
SCOOP12	P	V	R	S	S	Q	S	S	Q	A	G	G	R
SCOOP13	Y	L	P	P	S	K	S	R	K	G	K	G	P
SCOOP3	E	L	R	P	S	S	E	W	R	R	K	M	I
SCOOP8	D	F	E	G	S	I	S	G	Q	A	G	G	G
SCOOP10	F	T	G	P	S	G	S	G	H	G	G	G	R
SCOOP11	D	V	G	A	S	S	S	G	Q	G	G	G	R
SCOOP14	F	V	P	P	S	T	S	H	K	G	Q	G	P
	1	2	3	4	5	6	7	8	9	10	11	12	13

- non polar
- polar
- charged +
- charged -

Figure 25: **Amino acid composition of the 14 SCOOP peptides.** Peptides that showed an activity in the applied assays are indicated in white. Peptides without activity in red and peptides with partial activity in orange. The alignment of all 14 SCOOP peptides shows that most of the active peptides harbor a positively charged aa at position 6.

The SCOOP family contains several defense inducing peptides

Various peptides have been shown to effect and regulate root growth. As described in chapter 3 also PROSCOOP12 is a negative regulator of root growth (Figure 9). Moreover, we showed that PROSCOOP12 expression is induced by SCOOP12 and PROPEP2 expression is enhanced by SCOOP12 application (Figure 19). These results suggest a functional link between the two peptide families. A collaboration between different peptides has also been described for *AtPep1* and *PIP1* which act cooperatively to amplify triggered immunity. However, the SCOOP12 peptide has a similar effect on root growth inhibition like *AtPep1* but still seems to have additional effects on roots. The observed radial swollen root tips phenotype is commonly reported to be caused by salt, drought and osmotic stress (BursSENS et al., 2000; Ji et al., 2014). In case of these stresses, the root tip swelling is connected to a decrease of cell cycle activity in the root meristem. The decrease appears in the zone with *CycB1* expression. *CycB1* is associated with actively dividing cells (Ferreira et al., 1994). This indicates a shrinkage of the meristematic region. The short peptide RGF (ROOT GROWTH FACTOR) was shown to be an important factor for the maintenance of the root stem cell niche by restoring *CycB1* expression in a mutant with reduced root meristem size and loss of coordination between cell elongation and expansion in the elongation- differentiation zone. It is possible that the SCOOP peptide family members negatively regulate the expression of RGF. It would be interesting to apply the peptides to the *CycB1* reporter line to further investigate the lateral swollen root tip phenotype. Moreover, the strong secondary root growth could be explained by a cross-regulation of other peptide families with SCOOP peptides. Most of the RGF/GLV/CLEL gene family members are expressed during lateral root development (Fernandez et al., 2013). The overexpression of many of these gene family members results in a reduction of lateral root growth (Delay et al., 2013). It is possible that SCOOP peptides have expression promoting effect on certain members of these peptide families, like we could show for PROPEP2 (Figure 12). It would be interesting to investigate to transcription expression level of members of these peptide families after SCOOP peptide treatments. A strong pigmentation of plant tissue is usually observed during various stress conditions. Often the darkening of leaf tissue is connected to the accumulation of anthocyanin. In many plants the anthocyanin accumulation is induced by biotic and abiotic stresses (Brosché and Strid, 2002; Li and Strid, 2005). However, our observations were not made in shoot tissue and *Arabidopsis* roots usually do not show an accumulation of anthocyanin. However, a pigmentation of roots could be observed by low-sulfur conditions in *Arabidopsis* (Jackson et al., 2015). For the moment we cannot explain the

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root pigmentation. We cannot exclude a possible interference of SCOOP peptides with the uptake and metabolization of molecules like sulfur. Moreover, the exact localization of cells which show the pigmentation will require further investigations. In conclusion, we show that the PROSCOOP family contains several peptides with defense activating properties. Moreover, these peptides can have remarkable effects on root development and phenotype. The SCOOP peptides could therefore bridge the gap between small peptides involved in plant developmental processes and peptides mainly serving as DAMPs.

5.5. Material and methods

5.5.1. Plant material

Plant material used was wild-type *Arabidopsis thaliana* L. Heynh cultivar 6 Columbia (Col-0) as well as *bak1-5 bkk1-1* as described by (Roux et al., 2011) were grown under short day conditions (photoperiod of 8h light at 22°C/16h dark at 21°C, with 70% of relative humidity). Plants used for expression analyzation were grown under long day conditions (photoperiod of 16h light at 22°C/8h dark at 21°C, with 60% relative humidity).

5.5.2. Elicitor peptides

SCOOP elicitor peptides were used as described in table 2. As well as *AtPep1* (ATKVKAKQRGKEKVSSGRPGQHN) and *flg22*(QRLSTGSRINSAKDDAAGLQIA)

5.5.3. Seedling growth inhibition assay

Seedlings were germinated on MS agar and grown for 5 days before transferring one seedling per well to 24 well plates containing 500 µl MS media or MS media supplied with the indicated elicitor peptide to a final concentration of 1 µM (six replicates per elicitor peptide treatment). Photos were taken, fresh weight and root length were measures after 8 additional days.

5.5.4. ROS measurements

For ROS assays leaf discs of three weeks old soil grown plants, were placed into each well of a white 96-well plate (Thermo Scientific, Waltham, USA) in 0,1 ml of water and kept in the dark overnight. For elicitation and ROS detection, horseradish peroxidase and luminol were

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added to a final concentration of 10 $\mu\text{g ml}^{-1}$ and 100 μM , respectively. Luminescence was measured directly after addition of elicitor peptides in a FLUOstar OPTIMA plate reader (BMG LABTECH, Offenburg, Germany).

5.5.5. Determination of expression

Detached leaves of three weeks old plants were collected and floated for two hours in water supplied with the indicated elicitor or control solution. After the treatment, material was frozen and ground in liquid nitrogen. RNA from 100 mg of tissue was extracted using the NucleoSpin RNA plant extraction kit (Macherey-Nagel Hoerd, France). The DNase treatment was performed according to the manufacturer's recommendations. Per PCR reaction, complementary DNA was synthesized from 10 ng of total RNA extract with oligo(dT) primers using Moloney Murine Leukemia Virus Reverse Transcriptase according to the manufacturer's instructions (Promega). For quantitative real-time reverse transcription PCR (qPCR) in a 96-well format, the Chromo4™ System (Bio Rad) was used. Expression was normalized to that of the gene *ACR12* (AT5G04740) using the qGene protocol (Muller et al., 2002). All the gene-specific primers used are detailed in Table 4

Table 4: Primers used for experiments in this chapter

Name	Gene ID	Sequence forward	Sequence reverse
<i>PROSCOOP1</i>	AT5G44565	AGCATCCTCTTTCACCATACCG	ATTCTGACCACCACCACCTC
<i>PROSCOOP2</i>	NA	TAATTGTGCTGGTCTCATGCTC	GCGGTGGCGGCGGTTTTT
<i>PROSCOOP3</i>	NA	GGTCCTTTGAATTTGAGACTTTTG	TAATACGAGCTCTTCGACCATAC
<i>PROSCOOP4</i>	AT5G44568	ATCTCAAGTTGGAGTCGCC	TTATCTTTAGGCGATGCAGAGTGA
<i>PROSCOOP5</i>	AT5G44570	ATACAATCCACCGACGCTGC	GGATAGAGCATTGTGGCTGC
<i>PROSCOOP6</i>	AT5G44572	CTTGCAGCCTTAGCCAATCG	TCATCAATCTCCTCCCGTGG
<i>PROSCOOP7</i>	AT5G44574	CACTTGCCTTAGCGTAACGG	TGGTGAGTTTTCTCCACGCT
<i>PROSCOOP8</i>	AT5G44575	TCCCAACCCATACGGAGTCT	TTTGTTGACCACCACCGGC
<i>PROSCOOP9</i>	AT5G44578	TCCGTATCCGTATGGTGGCA	ATGCTGCTACCACCATGTCC
<i>PROSCOOP10</i>	AT5G44580	TGGGGAGGAAGCGGATGAAT	CACTGCCTGATGGTCTGTGA
<i>PROSCOOP11</i>	AT5G44582	TTGTAATCACTGGAAGGAGG	GTTGCGACCACCACCTTGT

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<i>PROSCOOP12</i>	AT5G44585	TTCTTCTCCTCTGCACCGTC	TAAAACGTCCACCAGCTTGG
<i>PROSCOOP13</i>	AT1G22885	TGATATCCTTTCAAGTTGGAGTCG	TTATGGACCTTTTCCTTTGCGC
<i>PROSCOOP14</i>	AT1G22890	CTCACAAGTTGGACTAGGCGA	GGGCCTTGTCTTTGTGTGA
<i>ACR12</i>	AT5G04740	TTGTTGATGATCGCCGGAA	TGGAACAACGTCGTCATCGT

5.6. Appendix: Are PROSCOOP genes under epigenetic control?

5.6.1. Introduction

Plants need to have the capacity to reprogram gene expression in order to respond rapidly to various stresses. Chromatin modifications play important roles in regulating gene expression and in transcriptional reprogramming. A lot of work was done on the influence of chromatin modifications and remodeling of defense-related genes (Berr et al., 2012). However, most studies focused on epigenetic modifications initiated by pathogen perception and hormone homeostasis changes upon defense signaling but less on epigenetic changes upon perception and signaling of small endogenous peptides. One of the widely discussed epigenetic change involved in plant resistance is the modulation of the chromatin structure. The histone tails can undergo diverse reversible post-transcriptional histone modifications. These modifications include acetylation, methylation, phosphorylation, ubiquitination, sumoylation, carbonylation and glycosylation (Kouzarides, 2007). The modifications can directly modulate the chromatin structure or promote the recruitment of specific effectors which determines the function of the chromatin modification and their functional outcome (Yun et al., 2011). Depending on their targets, histone methylation and/or ubiquitination can activate or repress transcription. One of the most intensely studied chromatin marks connected with gene repression is H3K27me3 (Roudier et al., 2011). Next to the influence of chromatin marks also the involvement of ATP dependent chromatin-remodeling enzymes as contributors to fast and reversible and/or heritable gene expression control is currently in focus of research in the plant innate immunity (Berr et al., 2012). The research on chromatin modifications and plant immunity focus mostly on histone modifications of hormonal pathways like salicylic acid and the jasmonic/ethylen mediated plant defense signaling. These signaling pathways are part of the multilayer defense system of plants, consisting of PTI and ETI responses, leading to SAR. The well-defined markers for SA-mediated basal and R gene-mediated defense signaling *PR1* and *PR2* genes are among the most intensely investigated genes regarding chromatin modifications. Several studies have shown that the activation of *PR1* is correlated with an increase in the level of acetylated histones at the *PR1* locus in *Arabidopsis* and tobacco (Butterbrodt et al., 2006; Mosher et al., 2006; Koornneef et al., 2008). The resulting induction of SAR by increased *PR* gene expression is often linked to

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priming for stronger activation of various defense responses that are induced following the attack by pathogens (van Hulten et al., 2006). Moreover, priming of innate immunity is correlated with chromatin modification of the promotor region of WRKY transcription factors as well as SA and PTI correlated genes (Jaskiewicz et al., 2011; Luna et al., 2011; Po-Wen et al., 2013). Regarding peptide triggered immunity much less is known. Recently it was shown that the histone methyl transferase SET DOMAIN GROUP (SDG8) and SDG25 regulate *AtPep1* and *flg22* triggered immunity, ETI as well as SAR. These two genes affect global and locus-specific H3K4 and H3K36 methylation leading to the regulation of plant immunity genes (Lee et al., 2016). In order to increase the knowledge of the influence of chromatin modification on small endogenous peptides we wanted to investigate the distribution of the chromatin mark H3K27me3 at PROSCOOP12 and PROSCOOP7. We choose these two members of the SCOOP family because we had a first identification, by chromatin immunoprecipitation sequencing (ChIP-seq) on WT seedlings, that these two genes might have an increased level of H3K27me3. In summary we show the influence of treatments with the recently discovered peptide SCOOP12, on the chromatin mark H3K27me3. Here we focus on the H3K27me3 chromatin mark present on the SCOOP peptide family members *PROSCOOP7* and *PROSCOOP12*.

5.6.2. Results and discussion

After we investigated the effects of the SCOOP peptides on plant immunity as well as the effect on root growth, we were interested in the expression regulation of *PROSCOOP*. We showed in chapter 5 that *PROSCOOP7* and *PROSCOOP12* expression was enhanced by treatments with SCOOP12 (Figure 19). Next, we investigated possible mechanisms involved in the feedback loop of the small peptide to its putative precursor. Our in-house ChIP-seq of H3K27me3 data on untreated WT seedlings revealed that this chromatin mark is widely distributed over SCOOP family members (Figure 26A). Especially in the promotor regions of *PROSCOOP1*, 6, 7 and 8 as well as in the gene body of *PROSCOOP12* a clear peak of H3K27me3 is noticeable. The genes *PROSCOOP2* and 3 are not annotated but present between *PROSCOOP1* and *PROSCOOP4* (Figure 26A). The H3K27me3 peak on the first exon of *PROSCOOP12* and the peak in the promotor of *PROSCOOP7* raised our interest. In order to investigate the changes of H3K27me3 level upon treatments we designed primers covering the DNA sequences of the H3K27me3 peaks on *PROSCOOP7* and *PROSCOOP12*. On

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PROSCOOP12 we designed three primer sets. Primer set 1 amplifies in the promotor of *PROSCOOP12*, while set 2 amplifies a region directly covering the H3K27me3 peak in the first exon and the set3 a region in the second exon (Figure **26B**). The primer sets 4 and 5 amplify regions at or before *PROSCOOP7*. Set 4 at the H3K27me3 peak in the promotor and set5 after the peak in the first exon of *PROSCOOP7* (Figure **26C**). Next, we decided to treat Arabidopsis plants with SCOOP12. The treatments were applied with the same experimental setup than used in Figure **19**. Chromatin of SCOOP12 treated and untreated control plants was extracted. After, an immunoprecipitation (IP) with a specific antibody for the H3K27me3 chromatin mark was performed. A real-time PCR of input and IP samples with the primer sets was performed and normalized to the expression of *18S*. Our results show that H3K27me3 is increased by SCOOP12 treatment at *PROSCOOP12* at the primer sets 2 and 3 as well as at set 4 on *PROSCOOP7* (Figure **26DE**). On *PROSCOOP12* at primer set 2 and 3 H3K27me3 is weakly increased by a fold change of 1.5 in comparison to the control (Figure **26D**). On *PROSCOOP7* set 4 H3K27me3 was found to be doubled upon SCOOP12 treatment (Figure **26E**). However, the data presented here are preliminary and miss a repetition and thus only provide a first indication that the expression of *PROSCOOP* genes might be influenced by specific chromatin marks. The results are rather unexpected since the presence of H3K27me3 in the promotor of genes is related to the repression of expression (Roudier et al., 2011). We show that the expression of *PROSCOOP7* and *12* is increased by SCOOP12 treatment. However, the H3K27me3 fold change is not strong in comparison to previous studies. It might be possible that the regions at the two genes are not representative and do not contribute to the expression regulation. Moreover, it is possible that other chromatin marks influence the expression of the *PROSCOOP* genes. In order to further investigate the role of chromatin modifications at *PROSCOOP* genes it would be necessary to add additional replicates and to test additional chromatin marks such as H3K4me3. Moreover, it would be interesting to investigate the influence of SCOOP12 on the whole genome chromatin marks of H3K27me3 distribution as well as the with gene expression promoting associated chromatin mark H3K4me3. These experiments would provide new insights into the epigenetic regulation of small peptide influenced gene expression.

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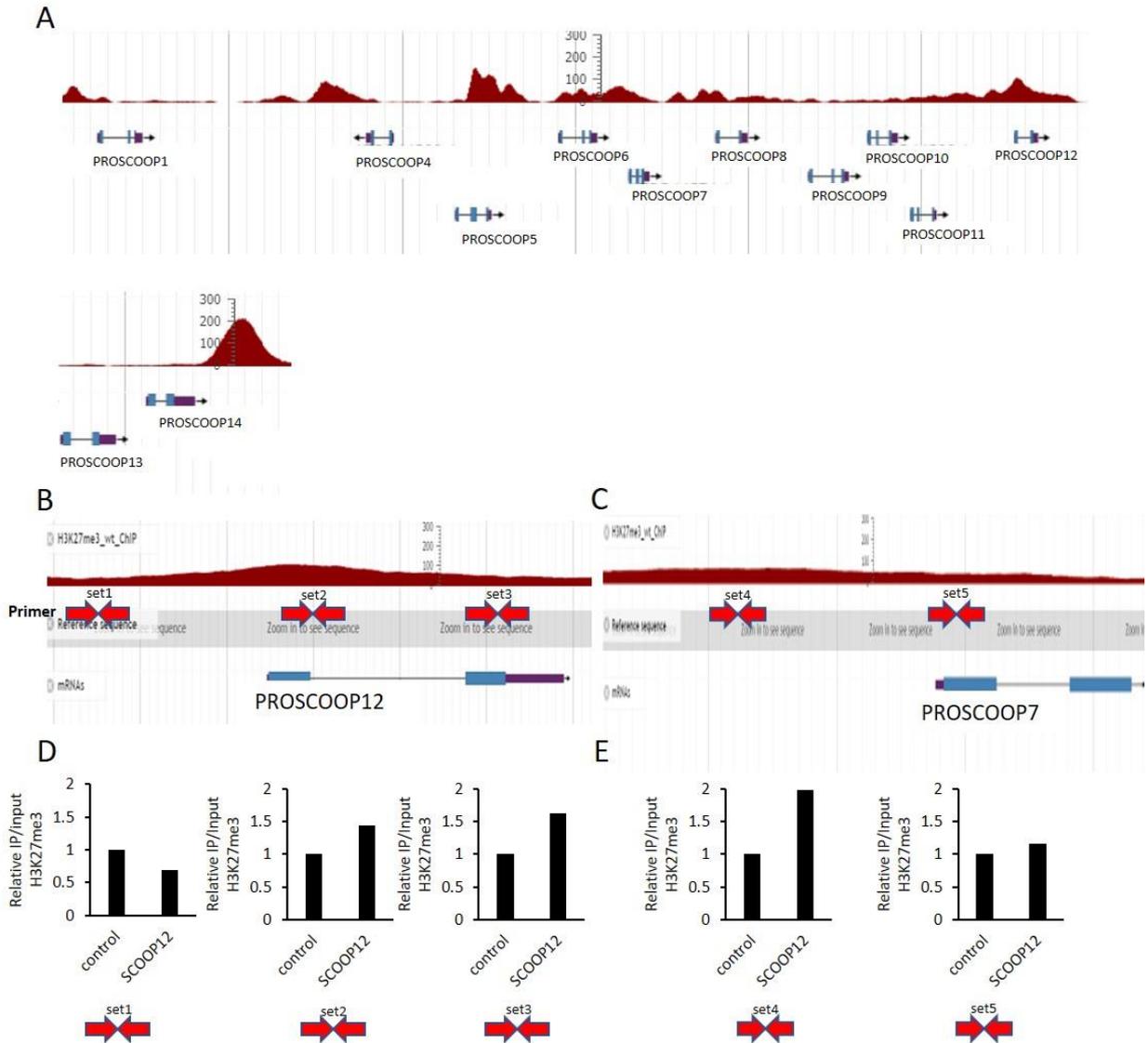


Figure 26: **H3K27me3 distribution at *PROSCOOP* genes.** A) Distribution of H3K27me3 chromatin mark over all members of the SCOOP peptide family. B, C) H3K27me3 distribution at the two SCOOP family members *PROSCOOP7* and *PROSCOOP12*. 3 Primer sets to investigate the level of H3K27me3 at *PROSCOOP12* and 2 primer sets for *PROSCOOP7*. D,E) ChIP-real-time PCR result of H3K27me3 IP after SCOOP12 treatment for all 5 primer sets. The real-time results are represented relative IP to input expression normalized to *18S*. The results show one biological replicate.

5.6.3. Material and methods

5.6.3.1. ChIP

For chromatin extraction we used an adopted protocol from (Ikeuchi et al., 2015). For sonication we used the M220 Focused-ultrasonicator (Covartis USA). Immunoprecipitation was performed with specific H3K27me3 antibodies (ab8580) (Abcam USA).

5.6.3.2. Real time PCR and specific primer

For quantitative real-time reverse transcription PCR (qPCR) in a 96-well format, the Chromo4™ System (Bio Rad) was used. The relative IP to input values are normalized to that of 18S (AT2G03810). The primer sequences are shown in table 5.

Table 5: List of primers used for ChIP coupled with real time PCR

Name	Sequence forward	Sequence reverse
18S	TCCCTTCACGGCCGGCTTCT	TCGCGGGCGGCGAACCAC
Set1	TTATGAAATGCTATGTACAACCGC	TAGAGGAATAACTGTAATAGAGCG
Set2	GTTCCCTCCACCTGCATGTA	AAACCGGTTAGTAGGACTGAATG
Set3	TCGATTTATATGTTTTGTTAATTTCTCA	GATCTTACTGGTCCCGAAGC
Set4	GTATGCATGAAATCGCCAGTTATA	CGATTCCAAGTTTAACTCCTTTTG
Set5	ACTCAGTTTTTCTCTCTGCTTGG	GAGCCGTTACGCTAAGGCAA

6. Biotic stress-induced priming and de-priming of transcriptional memory in Arabidopsis and apple

“Without forgetting it is quite impossible to live at all.”

Friedrich Nietzsche – German philosopher (1844–1900)

This chapter contains results of a project I started before we discovered the SCOOP peptides, as described in the previous two chapters. The intention of this project was to uncover the influence of synthetic plant defense inducing compounds on Arabidopsis and apple. Our

findings are rather unusual and have the potential to uncover an unknown mechanism of plant memory. My contribution to this chapter is the complete experimental part, data compilation and writing of the first manuscript draft as well as the data analyzation with help of Jean-Marc Celton, Alexandre Degrave, Marie-Noelle Brisset and Etienne Bucher. This chapter will serve as manuscript for a publication.

6.1. Abstract

Under natural growth conditions plants experience various and repetitive biotic and abiotic stresses. Salicylic acid (SA) is a key phytohormone involved in the response to biotic challenges. Application of synthetic SA analogues can efficiently prime defense responses and leads to improved pathogen resistance. Because SA analogues can result in long-term priming and memory, aimed at identifying genes with the potential to memorize treatments with an SA analogue and explored the role of DNA methylation in this memorization process. Here, we show that treatments with SA analogues can lead to long-term transcriptional memory of particular genes in Arabidopsis. We found that subsequent challenging of such plants with a bacterial elicitor reverted this transcriptional memory bringing their expression back to the original pre-treatment level. We also made very similar observations in apple (*Malus domestica*), suggesting that this expression pattern is highly conserved in plants. Finally, we found a potential role for DNA methylation in the observed transcriptional memory behavior. We show that plants defective in DNA methylation pathways showed a different memory behavior. Our work improves our understanding of the role of transcriptional memory in priming and has important implication concerning the application of SA analogues in agricultural settings.

6.2. Introduction

Plants are under continuous attack by pathogens because they are rich sources of nutrients. However, they protect themselves by physical barriers, such as a waxy cuticular layer or by protective periderm. In addition to these barriers plants have evolved an immune system

comprising constitutive and inducible defenses. The inducible immune system is based on the specific recognition of pathogen-derived molecules (Chisholm et al., 2006; Jones and Dangl, 2006a). This so-called pattern-triggered immunity (PTI) is achieved by plasma membrane localized pattern-recognition receptors (PRRs) which directly interact with highly conserved pathogen/microbe – associated molecular patterns (PAMPs/MAMPs) (Gomez-Gomez and Boller, 2002; Zipfel et al., 2004). One of the best-studied examples of a MAMP, that is capable of activating plant immunity, is the bacterial flagellin which is the major component of the bacterial motility organ (Macnab, 2003). The perception system of flagellin is widely conserved across the plant kingdom since most plants respond to flagellin (Felix et al., 1999). The fastest MAMP-triggered defense responses are typically induced within 5 min and decrease within a 30min time frame (Maffei et al., 2007). These events include the following plant responses: apoplastic alkalinisation, burst of reactive oxygen species (ROS), activation of mitogen-activated protein kinases (MAPKs) and intracellular calcium burst (Romeis et al., 2001; Asai et al., 2002; Boller and Felix, 2009). However, late responses are induced within several hours to days. Late defense responses include the accumulation of callose, the inhibition of growth, the differential expression of defense genes and the production of salicylic acid (SA) (Boller and Felix, 2009). In line with short and long-term defense responses, plants are also capable of inducing long-lasting systemic immunity. By local compatible or incompatible interactions, such systemic immunity can be initiated. This results in systemic acquired resistance (SAR). SAR-like responses can be induced by exogenous application of SA or SA analogues (White, 1979; Metraux et al., 1990; Ward et al., 1991). However, SA does not seem to serve as a mobile signal *per se* inducing immunity in uninfected distal tissues, while several other small molecules have been proposed to fulfil such a role (Shah and Zeier, 2013).

Defense-related stimuli enhance the capacity of plants to activate defense responses (Conrath et al., 2006; Beckers and Conrath, 2007). Exogenous application of SA and other benzoic acid derivatives have been shown to induce resistance of tobacco against *tobacco mosaic virus* (TMV) and to cause the accumulation of pathogenesis-related (PR) transcripts (White, 1979). This discovery paved the way for companies to identify more potent related compounds. These compounds are referred to as synthetic plant defense elicitors (Bektas and Eulgem, 2014). One of the most frequently used elicitors is benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester (BTH), which is commonly named acibenzolar *S*-methyl (ASM) and

which is commercialized by Syngenta (Cole, 1999; Friedrich et al., 2003). In Europe this compound is commercialized as “Bion”. Application of BTH has been shown to restrain downy mildew infections in vegetables and to control a range of fungal, bacterial and viral diseases of important crops like tomato, cucumber, broccoli, tobacco, melon, pear and apple trees (Scarponi et al., 2001; Zavareh et al., 2004; Pajot and Silué, 2005; Jiang et al., 2008). In apple, it was shown that application can limit the extent of fire blight disease, which is caused by the bacterium *Erwinia amylovora* (Brisset et al., 2000; Maxson-Stein et al., 2002). The SAR-inducing ability of compounds such as BTH is frequently associated with a primed state in which plants are able to ‘recall’ a previous infection or exposure to stress. Primed plants are therefore capable of responding more rapidly and/or effectively to a subsequent biotic or abiotic stress (Conrath et al., 2006). The molecular mechanisms behind priming are largely unclear. Priming has been associated with the accumulation of post-translational modification of cellular compounds. These compounds have important roles in signal transduction and/or amplification. In general, an accumulation or modification of these compounds does not activate a broad panel of plant’s defense responses (Conrath et al., 2006). Epigenetic regulation of gene expression is another widely discussed mechanism involved in defense priming. It has been shown that histone modifications at promoters of defense-related transcription factors such as *WRKY6*, *53* and *29* contribute to priming of gene expression by BTH (Jaskiewicz et al., 2011). An additional epigenetic regulation mechanism is DNA methylation. In plants, DNA methylation is present in all three possible sequence contexts (CG, CHG, CHH, whereas H is A, T or C) and has been shown to influence defense responses (Downen et al., 2012; López Sánchez et al., 2016). DNA methylation in the CG context can be maintained by DNA METHYLTRANSFERASE1 (MET1) and in all sequence contexts, it can be triggered by the RNA-directed DNA methylation (RdDM) pathway. In RdDM, one of the main players is NUCLEAR RNA POLYMERASE D1 (NRPD1), the largest subunit of RNA Polymerase IV (Pol IV), which plays a key role in the initiation of siRNAs production (Herr et al., 2005). Another emerging regulation of gene expression involves antisense transcripts. It has been previously reported that genes transcribed in sense orientation, can also be transcribed in antisense orientation. Antisense transcripts include partial or complete sequences complementary to other transcripts and are endogenous RNA molecules (Wang et al., 2005). They play an important role in various processes, including the adaptation to biotic and abiotic stresses (Terry and Rouzé, 2000). Antisense transcripts are widespread in both

prokaryotes (Wagner and Simons, 1994) and eukaryotes (Vanhée-Brossollet and Vaquero, 1998). Here we show that stresses can lead to long lasting transcriptional memory (priming) and that priming can be reversed (de-priming) by subsequent stresses. Furthermore, our results show that antisense transcripts contribute to transcriptional memory and that DNA methylation is required for proper stress response.

6.3. Results

6.3.1. BTH induces short- and long-term defense responses in Arabidopsis.

While it is known that bacterial elicitors such as flg22 induce various defense responses in numerous different plants, less is known about the action of synthetic plant defense elicitors such as BTH. Here, we tested the short and long-term responses of plants treated with BTH. One of the fastest plant defense response is the accumulation of ROS (Bolwell et al., 2002). Therefore, we first tested if a BTH treatment resulted in a detectable ROS burst in Arabidopsis. We found that BTH applied at a final concentration of 1mM induced a weak albeit significant (p -value < 0.05) ROS burst, whereas a control treatment with flg22 lead to a strong accumulation of ROS (Figure **27**). However, it was shown that a lower BTH concentration of 100 μ M does not induce a significant ROS burst (Tateda et al., 2014). The increased BTH concentration could explain these different results. (Figure **27A**). One of the long-term defense responses is the deposition of callose. As shown by (Kohler et al., 2002; Tateda et al., 2014) BTH induces a deposition of callose in leaf tissue. We also observed that application of BTH lead to a strong accumulation of callose in Arabidopsis leaves (Figure **27B, C**). Another long-lasting defense response is growth inhibition. Addition of bacterial elicitors to the growth medium was shown to result in a strong inhibition of growth (Krol et al., 2010). Our results indicate that repetitive spraying with BTH led to similar effects. Seven days after germination (dag) young plants were sprayed with BTH and then treated two additional times at three days intervals (Figure **27D**). Plants treated three times with BTH showed a reduction of fresh weight after a recovery phase of 8 days following the last BTH exposure in comparison to the water control (Figure **27E, F**).

Biotic stress-induced priming and de-priming of transcriptional memory

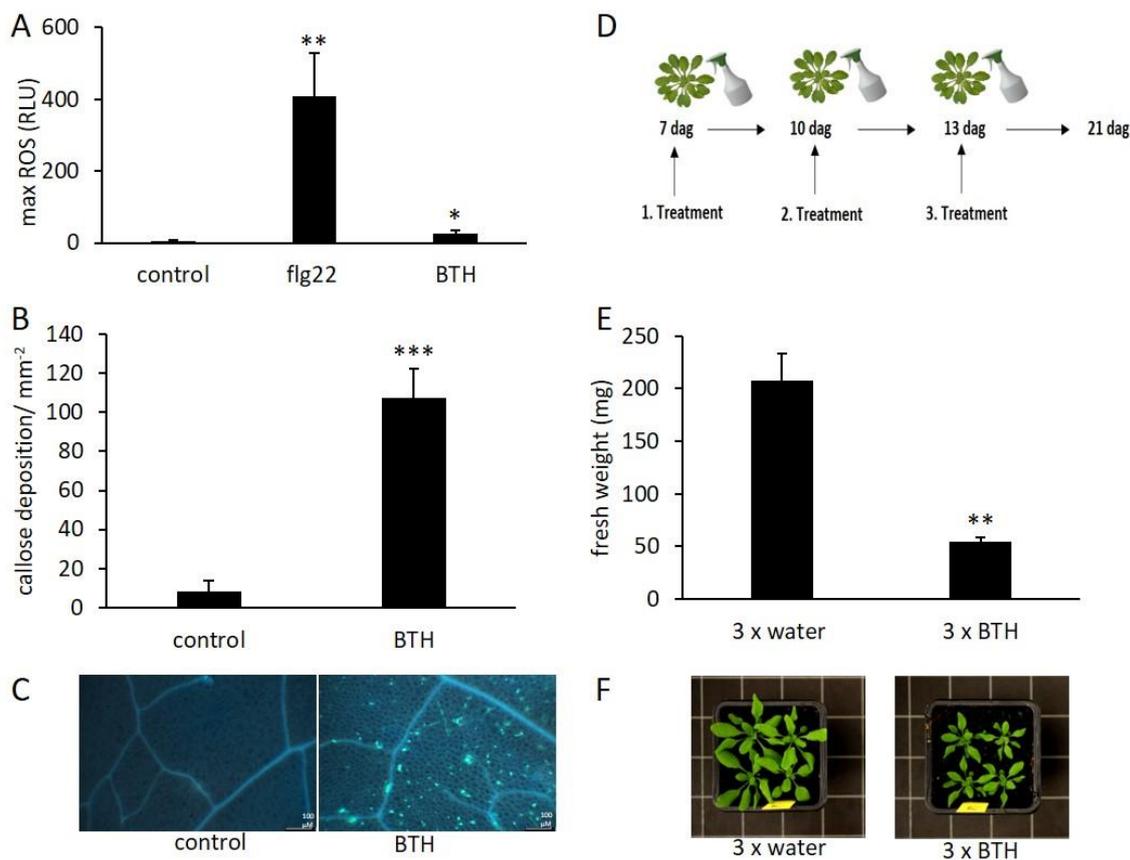
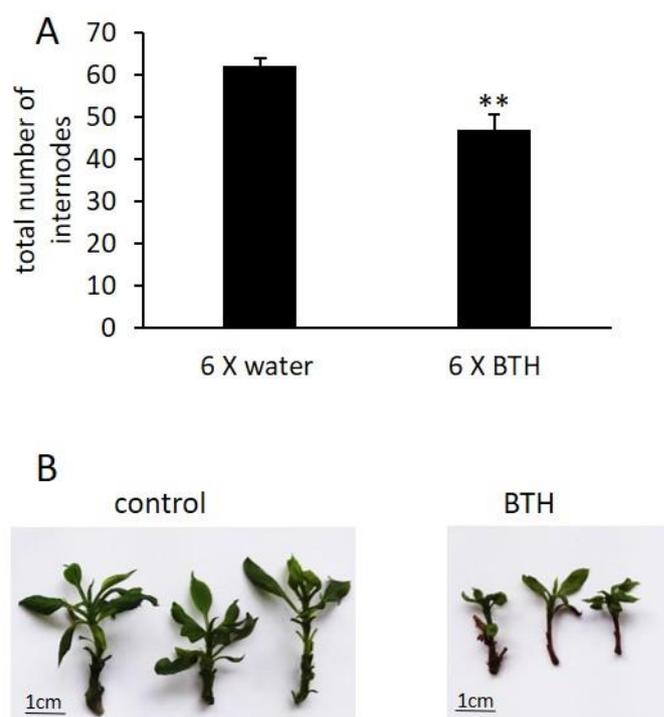


Figure 27: **BTH induces short- and long-term defense responses in Arabidopsis:** A: Production of reactive oxygen species (ROS) measured in RLU (relative light units) in wild-type Arabidopsis leaf-discs (Col-0), treated with 1 μ M flg22, 1mM BTH or without elicitor (control). Graphs display averages of 12 replicates B: Quantification of callose deposition. The bars represent the mean of 4 replicates. C: Localization of callose deposition by aniline blue staining. D: Temporal order of applied Arabidopsis treatments. The first treatment was applied 7 dag (days after germination), the third treatment 13 dag. E: Quantification of fresh weight of 21 dag old Arabidopsis plants. Plants were previously sprayed three times with water or BTH (1mM) according the scheme shown in D. F: Pictures of 21 dag old plants previously sprayed with water or BTH (1mM). Error bars show \pm SE of the mean. Significant differences according to Student's t-test results: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

To test the more general effect of BTH on other plants, we choose to investigate the growth repression effect, observed in Arabidopsis, on apple grown under two different growth conditions. Grafted apple plants exposed six times to BTH showed a lower number of internodes in comparison to a mock treatment (Suppl. Figure **28A**). Moreover, *in-vitro* apple plantlets grown for 4 weeks on media supplied with BTH showed a strong inhibition of growth (suppl. Figure **28B**).



(Supplementary) Figure 28: **Growth repression effect of BTH on grafted and in vitro apple plants.** A: Total number of internodes of grafted apple plants treated six times with BTH (1mM) or water. Two months after grafting, plants were treated every three days. Number of internodes was counted three days after the last treatment. Bars represent the mean of at least two biological replicates. B: Pictures of in vitro grown apple plantlets. After propagation plantlets were grown for 4 weeks after propagation on media supplemented with or without 1mM BTH. Error bars show \pm SE of the mean. Significant differences according to Student's t-test results: **, $P < 0.01$.

6.3.2. Transcriptional response and memory resulting from BTH treatment in Arabidopsis

BTH is associated with inducing a primed state of gene expression. In order to identify primed (and memorized) genes resulting from BTH application, we performed a series of microarray-based transcription profiles. Additionally, we investigated the role of a subsequent biotic stress exposure on BTH-induced long term transcriptional memory. To investigate this, we applied a series of treatments by spraying young Arabidopsis plants 7 dag. With a first application we primed gene expression with BTH. After a 3 days recovery phase, 10 days old plants were treated with the bacterial elicitor flg22. 11 days following the last treatment, plants were harvested and RNA extracted for subsequent gene expression profiling using two independent replicates (Figure 29A). Four different treatment combinations were used: A control treatment where plants were sprayed two times with water (w) (sample name: ww). Three combinations whereby either BTH (b) or water (w) was used in the first treatment and

water or flg22 (f) in the second treatment (sample names: bw, wf and bf, respectively, Figure **29B**). Gene expression changes induced by these treatment combinations were assessed using microarrays. The long-term effect of flg22 or BTH was investigated by comparing these samples against ww (ww Vs. wf and ww Vs. bw, respectively). To determine the effect of flg22 after previous BTH application we compared the samples bw against bf. The effect of BTH before a subsequent flg22 application was determined by comparing wf against bf. The impact of both compounds in succession was studied by comparing ww against bf (Figure **29C**). In order to facilitate this complex analysis, we decided to sort differently expressed transcripts (DETs, which include antisense transcripts) by using a binary code. For that purpose, every DET was assigned to a four digits number code. These four digits represent the examined microarray comparisons. The first position in the number code represents the comparison ww Vs. wf, the second ww Vs. bw, the third bw Vs. bf and the fourth the comparison wf Vs. bf. A 1 in the binary code indicates the presence of DETs in the corresponding comparison, while a 0 the absence (Figure **29D**). This binary code allowed us to provide a global overview of the different comparisons. By applying this binary code, we sorted all DETs into 16 categories (Figure **29E**). The category with the binary code 1000 contains transcripts that are exclusively differently expressed in the comparison ww Vs. wf and not in any other comparison. The category 1100 contains genes that are differently expressed in the comparisons ww Vs. wf and ww Vs. bw but not in the other two comparisons. Transcripts that are differently expressed in all four comparisons can be found in the category 1111 (Figure **29D** and **E**). This categorization of DETs revealed that a substantial number of transcripts are only differently expressed in one comparison and not in any of the others. Indeed, the categories 1000, 0100, 0010, 0001 contained between 2107 DETs (for 0010) and 4428 DETs (for 0001), respectively. 110 transcripts were found to be differently expressed in all four comparisons (Figure **29E**, category 1111).

BTH treatment (Figure 30A). Next, we investigated DETs, present in the category *11XX* in greater detail. Therefore, the category was divided into two sub-categories which are up- and down-regulated by BTH. Indeed, only 11.7% of the 909 transcripts upregulated by BTH (*11XX up by BTH*) were down-regulated by *flg22* thus showing a transgressive expression profile. We observed a similar trend for the BTH down-regulated transcripts (*11XX down by BTH*) where only 3.1% are transgressive. Both sub-categories show a similar distribution of sense and antisense transcripts, where 25% of all DETs are antisense transcripts (Figure 30B).

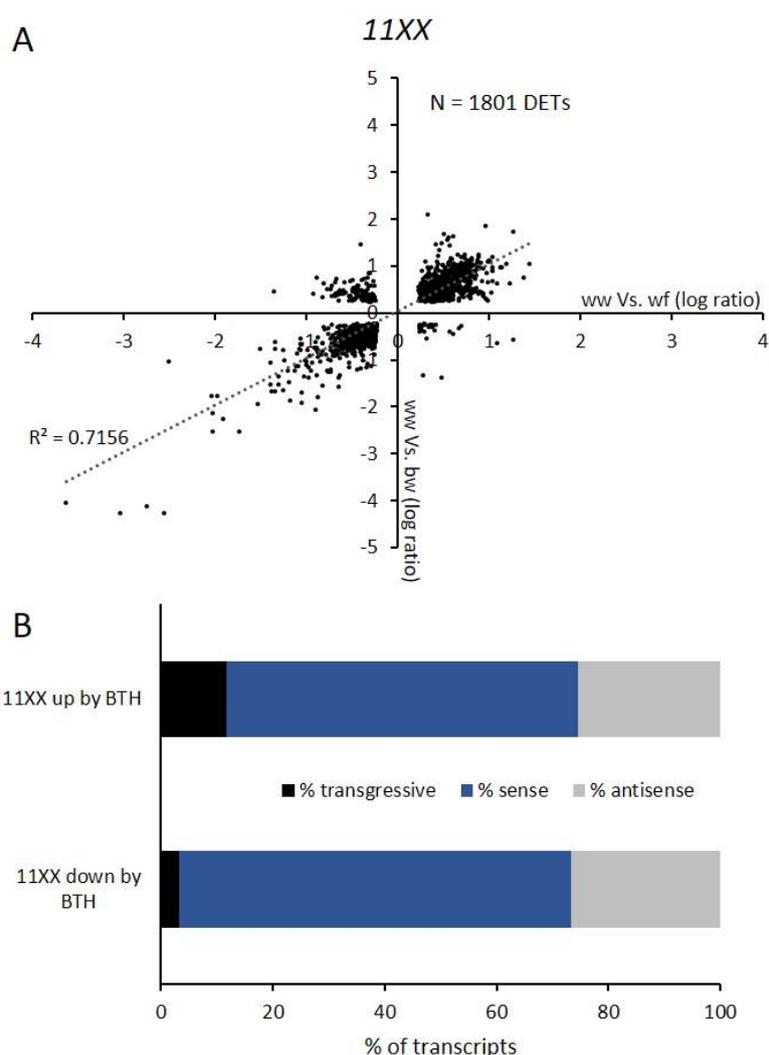


Figure 20: **Common differentially expressed transcripts resulting from *flg22* and BTH treatments.** A: Scatter plot of the log₂ expression values of *11XX* DETs showing *ww Vs. wf* on the X axes and *ww Vs. bw* on the Y axes. B: The category *11XX* was divided into *11XX up by BTH* and *11XX down by BTH* subcategories. The graph represents the percentage of transcripts expressed in sense and antisense in both sub-categories as well as transcripts showing transgressive expression pattern and therefore do not follow the global expression trend.

Next, we investigated the impact of subsequent stresses on BTH-induced transcriptional priming. The category 0110 contains genes that are differently expressed by applying BTH alone and by applying flg22 after a previous BTH exposure (ww Vs. bw and bw Vs. bf, respectively). 541 DETs were found in this category. The expression profile of these transcripts caught our attention because it showed that transcripts that were initially up-regulated by BTH were down-regulated by a subsequent flg22 exposure. Conversely, transcripts that were first down-regulated by BTH were up-regulated by subsequent flg22 treatment (Figure **31A**). Only 2.6% of all transcripts in this category show a transgressive expression profile (Figure **31B**). This suggested that subsequent stresses could erase the primed state of certain DETs. Therefore, we investigated how many of those 0110 DETs went back to a basal expression level. Because the application of BTH is closely related to the priming of genes (Katz et al., 1998; Kohler et al., 2002) (and this study) we tested if a subsequent flg22 treatment lead to a de-priming of genes. Here, we define de-priming as a primed transcript expression returning to a basal (pretreatment-like) expression level by a subsequent stress. To assess the number of de-primed genes, we compared the transcription level of DETs of category 0110 with that of DETs of the comparison ww Vs. bf (Figure **29C**). DETs of the category 0110, which are not significantly differently expressed in ww Vs. bf can be considered as de-primed. Out of the 541 DETs of category 0110 86.3% are not significantly differently expressed in the comparison ww Vs. bf anymore (p-value > 0.05) (Figure **31B**). Only 11.1% of these transcripts maintained their priming state after the flg22 challenge.

Closer inspection of the DETs in category 0110 revealed an uneven distribution of sense and antisense DETs. We investigated the two sub-categories of this category. The sub-category 0110 up by BTH contains DETs that are up regulated by BTH and show globally the inverted expression by subsequent flg22 treatment. The sub-category 0110 down by BTH contains DETs down regulated by BTH and up regulated by subsequent flg22 treatment. While transcripts in the sub-category 0110 up by BTH consist of 52.4% antisense transcripts, transcripts in 0110 down by BTH only contain 18.7% antisense transcripts and 80.2% sense transcripts (Figure **31C**).

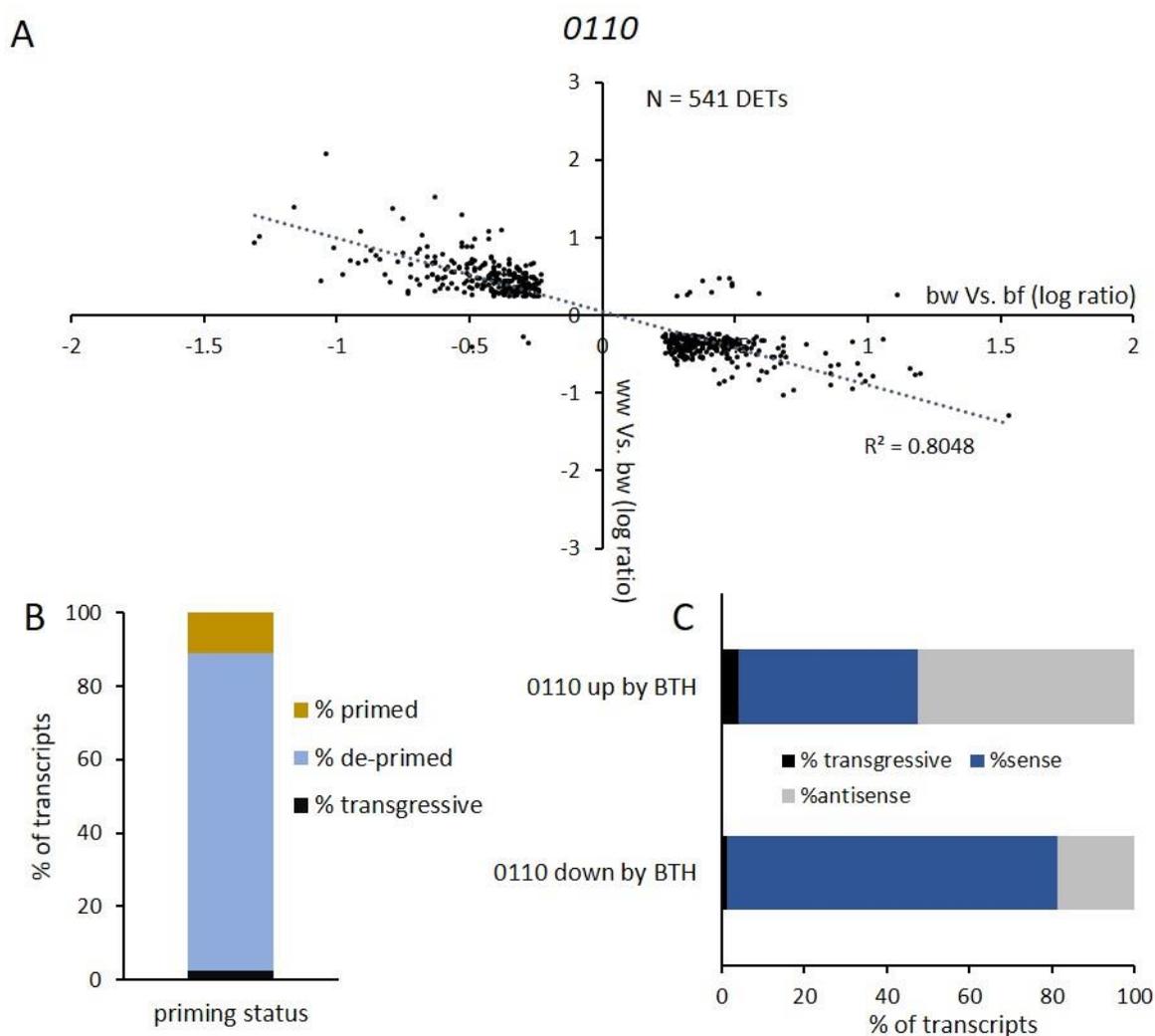
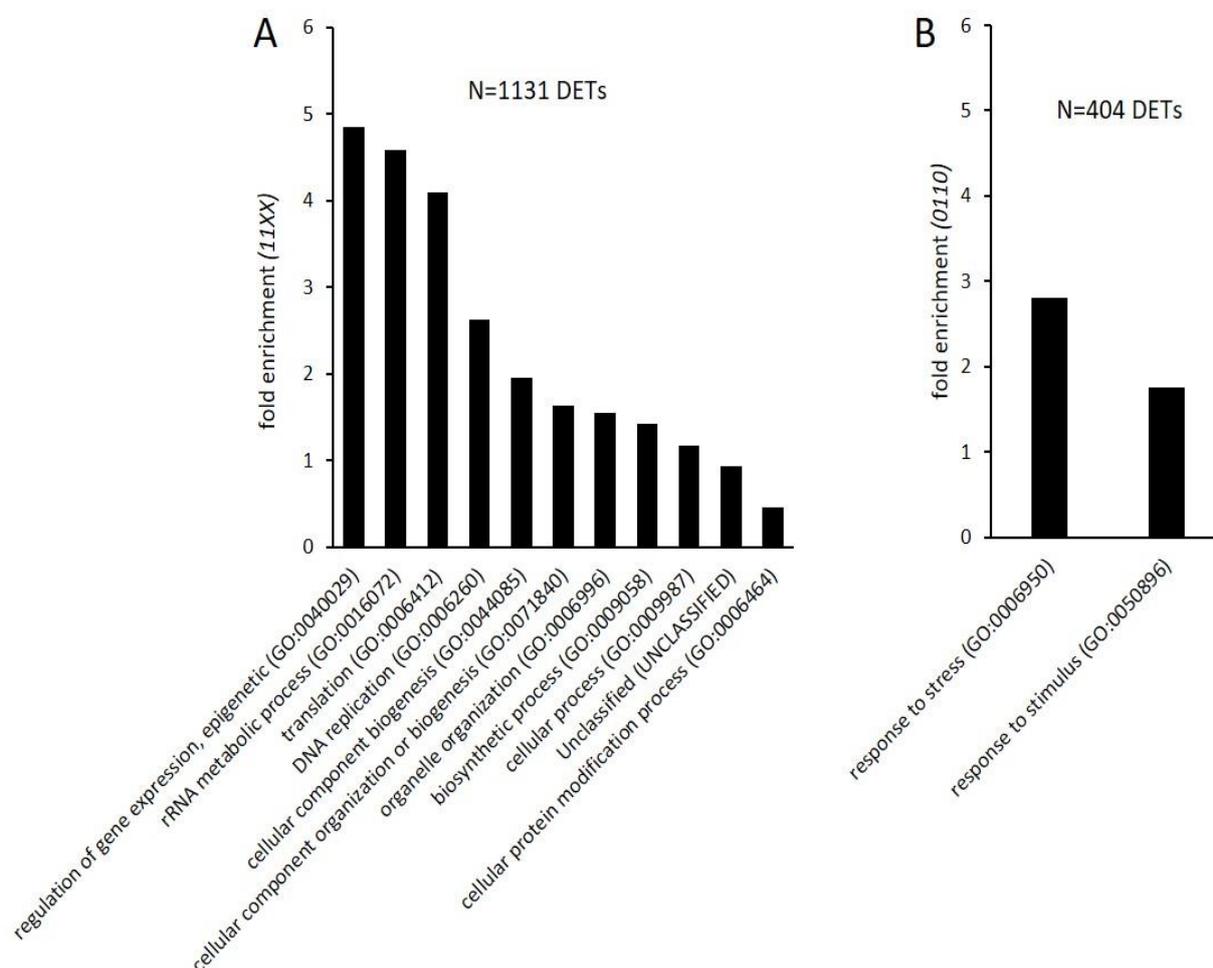


Figure 31: **DETs of the categories 0110 show an inversion of gene expression profile by a second stress.** A: Scatter plot of transcripts present in the category 0110. The category contains DETs of the comparison ww Vs. bw (Y axes) and bw Vs. bf (X axes). B: Classification of transcripts present in category 0110. The 541 transcripts were compared to DETs of ww Vs. bf. 86.3% of the transcripts are not differently expressed by the combination of both treatments and are considered as de-primed. 11.1% are differently expressed and therefore primed and 2.6% do not follow the global expression trend in 0110. C: Distribution of sense and antisense transcripts in the sub-categories 0110 up by BTH and 0110 down by BTH. The sub-category 0110 up by BTH contains 52.4% antisense transcripts.

Next, we studied the gene ontology overrepresentation of DETs in the category 11XX and 0110 (antisense transcripts included). DETs in the category 11XX show an overrepresentation in gene ontology correlated with the regulation of gene expression and epigenetics, rRNA metabolic process and translation (Supplementary figure 32A), while DETs in category 0110

are only overrepresented in the gene ontology response to stress and stimulus compared to the reference (Supplementary figure **32B**).



(Supplementary) Figure 32: **Gene ontology analysis of DETs in category 11XX and 0110. A:** PANTHER Overrepresentation test of DETs in category 11XX. Gene ontology was available for 1131 out of the 1801 DETs. DETs correlated with the regulation of gene expression and epigenetics (GO:0040029) are overrepresented with 4.85 fold enrichment compared to the Arabidopsis reference. **B:** Overrepresentation test of category 0110. Gene ontology for 404 of the 541 DETs were available. DETs correlated with the response to stress and stimulus (GO:0006950, GO:0050896) are overrepresented in this category. Gene ontology overrepresentation test was probed using the PANTHER webtool using a fisher`s exact test (Mi et al., 2017)

6.3.3. Transcriptional response and memory of stress treatments in apple

Next, we wanted to test if our observations in Arabidopsis were also relevant for other plant species. For that purpose, we performed a similar treatment regime as for Arabidopsis on *in vitro* grown apple plantlets followed by microarray-based expression profiling using the latest

version of the apple genome (Daccord et al., 2017). Plants were treated 14 days after propagation (dap) and 17 dap. After 14 days of recovery plantlets were harvested, RNA extracted and subjected to transcription profiling (Figure 33A). In total 3 different treatment combinations, on two independent biological replicates were applied (Figure 33B). Similar to our experiments in Arabidopsis we explored the effect of BTH as a first treatment (ww Vs. bw) as well the influence of flg22 application after BTH treatment (bw Vs. bf). In order to identify DETs that returned to a basic expression level, we investigated the effect of a combination of BTH and flg22 treatment on apple plants (ww Vs. bf) (Figure 33C). In the comparison ww Vs. bw 3920 DETs were detected. Notably, these DETs result from a treatment 17 days before harvesting and RNA extraction. In the comparison bw Vs. bf 847 DETs were found. 342 DETs are common in the comparison ww Vs. bw and bw Vs. bf. These common DETs are equivalent with the category 0110 of the Arabidopsis setup.

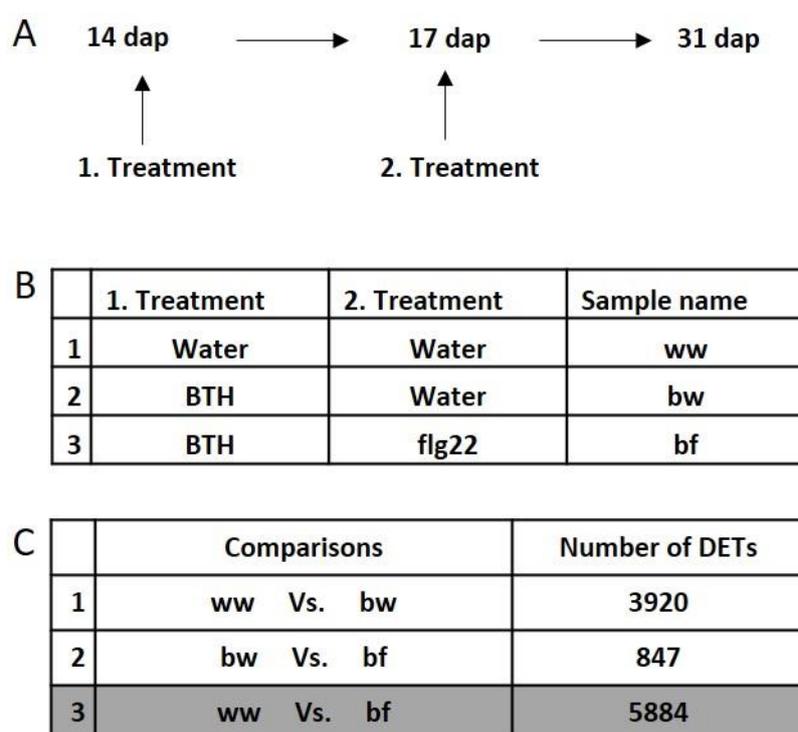


Figure 33: **Whole genome expression profiling and applied comparisons in apple.** A: Experimental set up of apple plantlet treatments. Apple plantlets were treated the first time 14 dap (days after propagation) followed by one additional treatment 17 dap. Leave tissue was harvested 31 dap. B: Combinations of applied treatments and sample names. C: List of examined microarray comparisons and number of DETs.

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The plot of the 342 common DETs confirmed our observations in Arabidopsis (Figure **34A**). In total, 82.7% of these transcripts are de-primed and only 8,2% are still significantly differently expressed and therefore primed (Figure **34B**). 9.1% of the transcripts are transcribed in a transgressive way (Figure **34B**). Next, we determined how many transcripts lost their priming status by comparing the common DETs with the comparison ww Vs. bf (Figure **34C**). However, for apple an overrepresentation of antisense transcripts such as we have seen for Arabidopsis was not observed. The subcategory of common DETs which are up regulated by BTH showed 47.3% antisense transcripts and DETs down regulated by BTH are expressed with a 57.1% antisense contribution (Figure **34C**). In total, common DETs have a higher content of antisense transcripts than found in our Arabidopsis comparisons. This observation is in line with a previous report that apple has globally a high percentage of antisense transcripts (Celton et al., 2014).

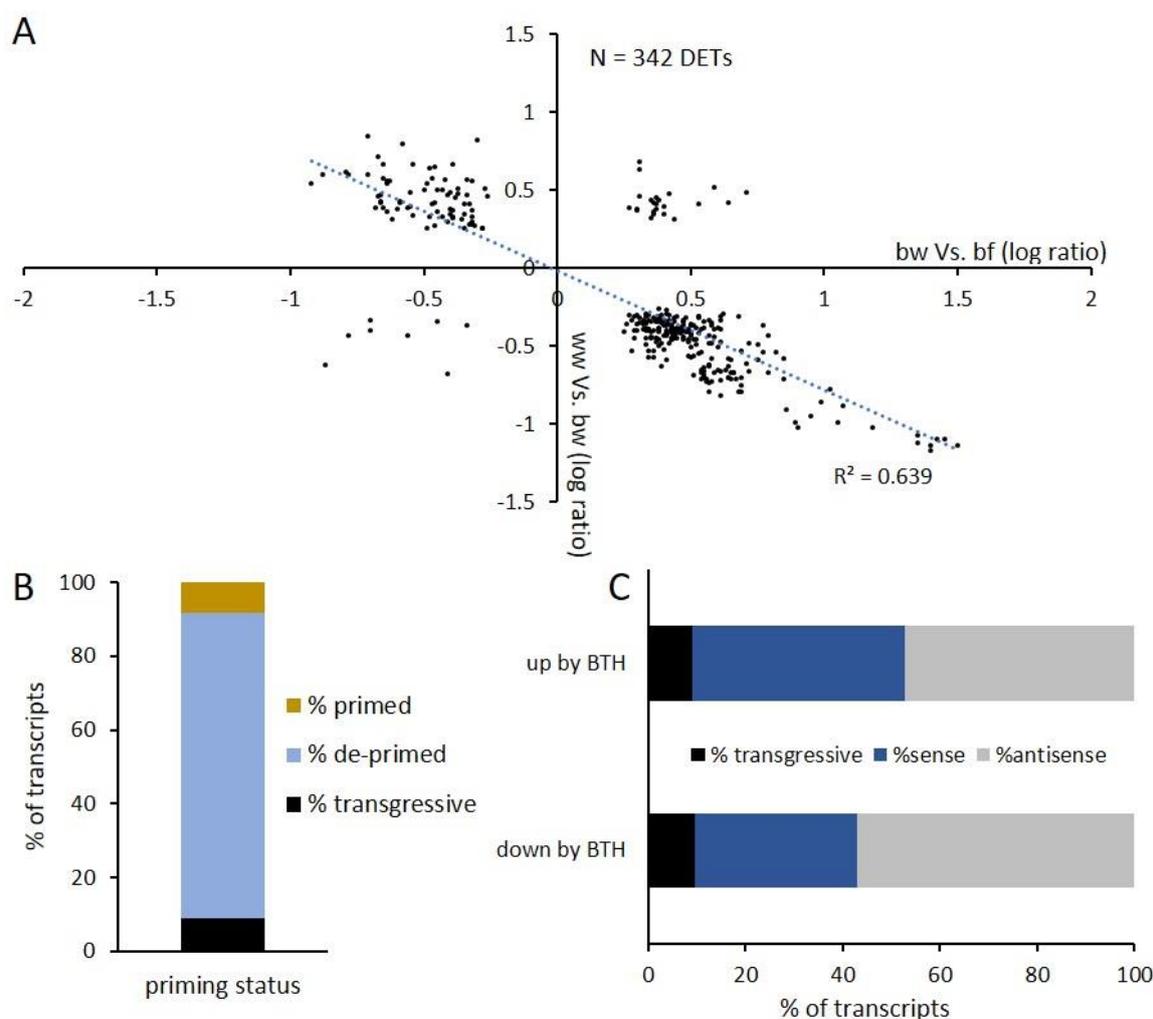


Figure 34: **De-priming in apple.** A: scatter Plots of common transcripts present in the comparisons ww Vs. bw (Y axes) and bw Vs. bf (X axes). B: The common transcripts are compared to ww Vs. bf. 82.7% are de-primed, 8.2% stay primed and 9.1% are transgressive and do not follow the global trend. C: The common DETs are divided into the sub-categories up by BTH and down by BTH

6.3.4. DNA methylation and de-priming of gene expression

To investigate the mechanism behind the de-priming of genes by a second stress, we chose to follow the expression profile of *AMY1* (*At4G25000*), a gene found in the Arabidopsis sub-category *O110 up by BTH*. To enhance the contrast resulting from the treatments on Arabidopsis we added a third treatment at 13 dag and harvested at 21 dag (Figure 35A). *AMY1* has previously been shown to be induced by biotic and abiotic stress and play an important role in starch metabolism (Stanley, 2002; Doyle et al., 2007). Again, to be able to measure the long-term memory on *AMY1* transcription, sampling was performed eight days after the last treatment. In the sample bbf *AMY1* was found to be significantly down-regulated in

comparison to the bbw treatment showing that we can follow de-priming by qPCR. Three BTH treatments (bbb) further enhanced the expression of *AMY1* in comparison to bbw and bbf (Figure **35B**). However, we could not detect a significant differential expression of *AMY1* in the sample bbw and bfw in comparison to the water control treatment. The expression profile of *AMY1* confirms the previous observations with respect to the enhanced expression of this gene by BTH and de-priming upon a subsequent flg22 exposure after BTH. Therefore, we used *AMY1* as a marker gene to investigate the molecular mechanisms involved in de-priming gene expression. To test if DNA methylation was involved in the memory or de-priming process, we applied the same combinations of treatments to *nrrpd1-3* and *met1-3* plants. For *nrrpd1-3*, the expression profile is similar to that of wild type plants. However, the amplitude is four times lower for all samples (Figure **35C**). The *met1* mutant did not show a significant response to any of the applied treatments, suggesting that *AMY1* activation and priming may directly or indirectly depend on DNA methylation.

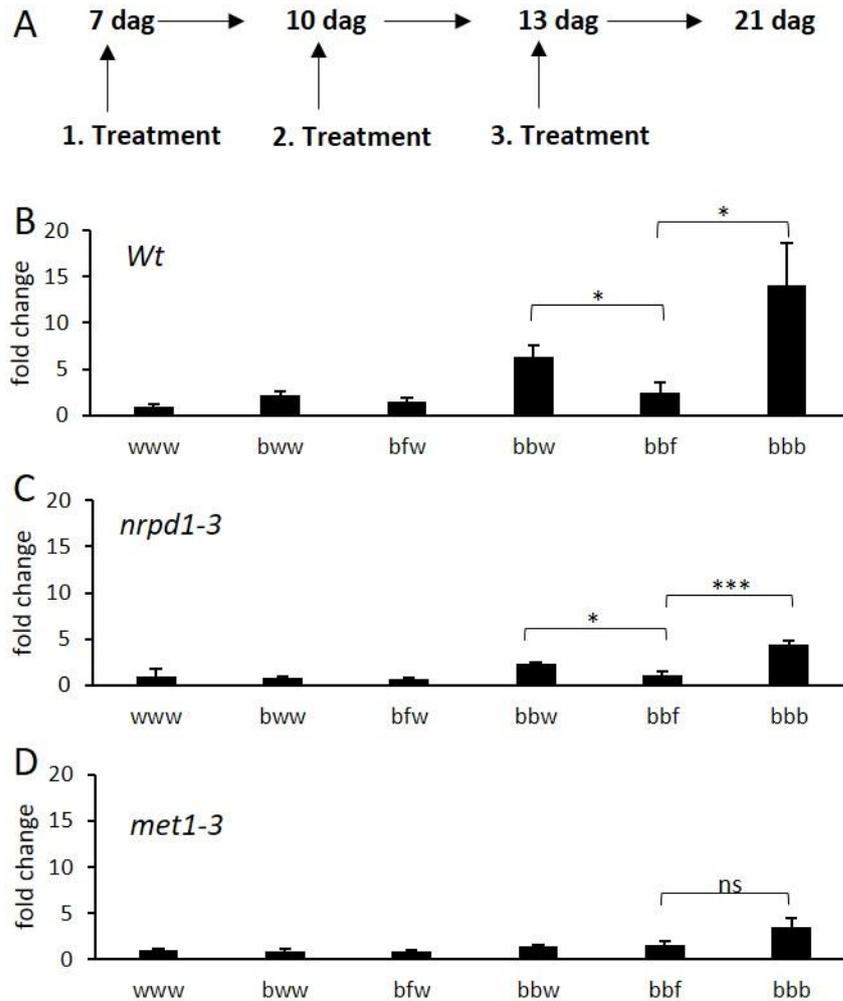


Figure 35: **Expression of *AMY1* serves as marker for de-priming of transcription.** **A:** set-up of treatments applied to Arabidopsis plants. The first treatment was applied 7 dag (days after germination) followed by two additional treatments. Plants were harvested 21 dag. **B:** Expression profile of *AMY1* by different sequences of treatments of wild type plants. **C, D:** The same treatment orders are applied to *nrpd1-3* and *met1-3*. All expression values are normalized to that of the gene *ACR12* (AT5G04740). Bars represent the mean of at least four biological replicates. Error bars show \pm SE of the mean. Significant differences according to Student's t-test results: *, $P < 0.05$; ***, $P < 0.001$; ns: not significant.

6.4. Discussion

6.4.1. BTH could have negative effects on plant vitality in an energy trade-off balance

In their natural environment plants are continuously exposed to a multitude of variable stresses. In an agricultural setting, plants such as apple need to be protected from various

diseases with pesticides since the lack thereof may be causing tremendous declines in yield. In order to reduce the use of pesticides, compounds are now being developed that can enhance natural pathogen defense mechanisms in plants. Because BTH can contribute to defense priming (Jaskiewicz et al., 2011; Conrath et al., 2015) (and this work) it could therefore have the potential to reduce yield losses. Here, we show that the application of BTH induces callose deposition and a ROS burst in Arabidopsis (Figure **27A, B**). While other reports did not see this (Tateda et al., 2014), this might be due to the tenfold higher BTH concentration of 1mM final concentration that was used in our assay. The induction of defense responses is widely connected to loss of energy in a trade-off balance (Walters and Heil, 2007). In line with the results we show here, the application of BTH induced an inhibition of growth in both, Arabidopsis and apple plants ((supp.) Figure **28**). These results indicate that BTH application at a higher concentration could have negative effects on plant growth.

6.4.2. De-priming of transcription is tightly regulated

With the series of microarray experiments performed here, we found that flg22 and BTH treatments globally result in similar transcriptional changes in Arabidopsis. The category *11XX* (Figure **30**) contains transcripts that are differently expressed by flg22 and BTH treatments, these transcripts show the same trend in expression as well as sense and antisense transcript distribution. BTH is an analogue of SA that is naturally produced by the plant (Bektas and Eulgem, 2014). SA is an important plant hormone that plays a role in the signaling pathway following flg22 perception (Yi et al., 2014). Therefore, it is reasonable that common deregulated transcripts resulting from the two different treatments globally show a similar expression profile in Arabidopsis. We then tested how a subsequent stress affects BTH-induced transcriptional memory. We show that, a flg22 treatment after BTH reverses the transcriptional memory in Arabidopsis (Figure **31A**) and in apple (Figure **34A**) of certain DETs. We termed such effects de-priming. It has previously been shown that BTH pre-treatments resulted in increased transcription of a subset of genes upon a second stress (Conrath, 2011; Jaskiewicz et al., 2011). Indeed, a similar de-priming phenomenon was observed for genes responding to repetitive drought stress. (Liu et al., 2014) showed that a subset of dehydration stress-response genes reacted to a first stress but did not respond to a second stress and stayed at a basic non-stressed expression level. These genes are referred as `revised-response` memory genes. Together with a follow-up work it was shown that the transcription

factor *MYC2* plays a critical role for gene activation upon a second drought stress (Liu et al., 2014; Liu et al., 2016). These results indicate that the regulation of de-priming in plants could depend on precise regulation of such a single gene. With our work we show that the number of de-primed transcripts by a subsequent exposure to a second biotic stress reflects the majority of commonly regulated transcripts (Figure **31B**, Figure **34B**). It is remarkable that we only find 2.6% of DETs in the Arabidopsis category *O110* and 9.6% of DETs for apple which show a transgressive expression profile and do not follow the global trend of de-priming (Figure **31B**, **34B**). Therefore, the number of transgressive DETs is low. Notable is also that the total number of DETs of the comparison bw Vs. bf in Arabidopsis and apple respectively: this comparison represents the lowest number of DETs in comparison to all other examined microarrays of both plant species (Figure **29C**, **33C**). This indicates that the global level of transcription is reduced by the subsequent flg22 treatment after BTH exposure.

6.4.3. DNA methylation could contribute to the priming properties of BTH

Two plant memory mechanisms have been proposed: The accumulation of proteins or transcription factors is one of them, epigenetic changes is a second potential mechanism (Bruce et al., 2007). Our results show a strong effect on priming and de-priming by global methylation decrease. We identified *AMY1* in the Arabidopsis category *O110* as a good marker for transcriptional memory because: (1) It has been shown that *AMY1* is a secreted protein that is expressed following biotic and abiotic stresses (Doyle et al., 2007), (2) it shows a memory accumulating property following multiple treatments (Figure **35B**) and (3) of its de-priming property upon a succession of BTH and flg22 treatments (Figure **35B**). Notably, we found that *AMY1* activation is strongly reduced in *met1-3* only being activated after three subsequent BTH applications. This suggests that DNA methylation may be required directly or indirectly to activate and/or prime *AMY1*. However, due to the weak activation of *AMY1* in *met1-3*, the de-priming effect of flg22 after BTH treatment could not be observed with this method (Figure **35D**). We thus cannot conclude on whether MET1 is required for the activation or the maintenance of the primed state of *AMY1*. In *nrrpd1-3* we found a low expression value but still detected the de-priming expression profile, indicating that DNA methylation via the RdDM pathway may be required to achieve the full potential of BTH treatments. Therefore, DNA methylation may be contributing to the priming and de-priming events which will have to be more closely investigated in the near future.

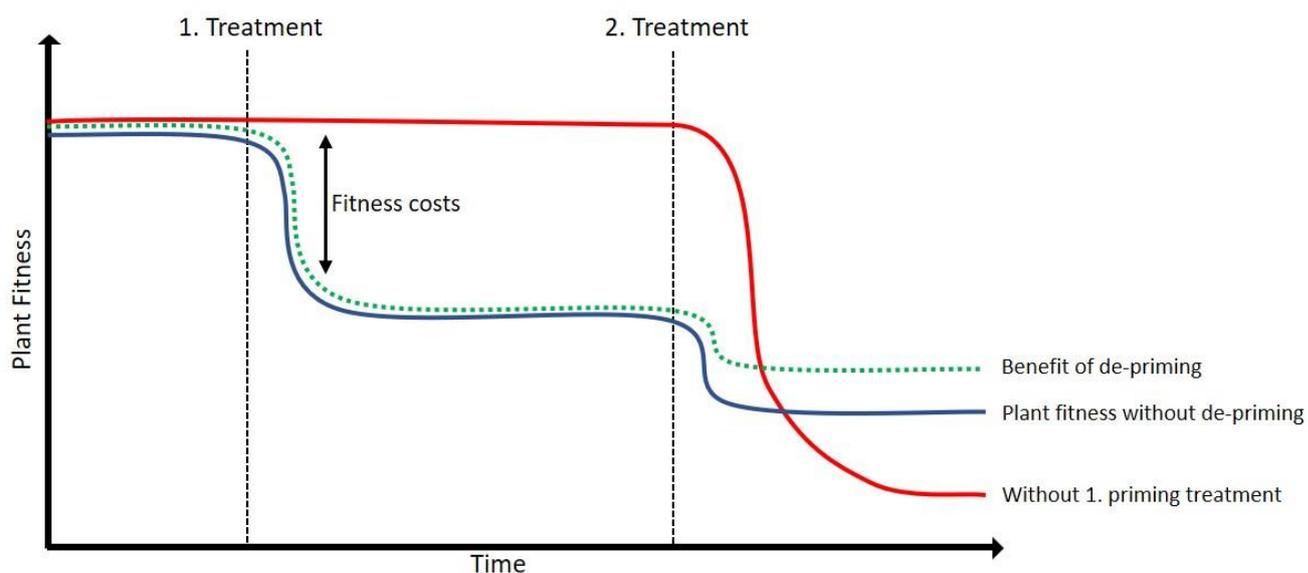


Figure 36: **Proposed model of de-priming effect on plant fitness.** Here we suggest a model of a possible beneficial effect of transcriptional de-priming. If plants are not exposed to a first priming treatment the second treatment might cause stronger deficits on plant fitness (red line) in comparison to plants that are primed (blue line). However, the priming reflects a fitness costs for plants due to the induced defense response and the maintenance of the primed transcription and/or epigenetic memory. The second treatment could cause a less pronounced fitness cost in comparison to the un-primed plants. We propose that the de-priming of a DET subset could lead to an additional positive effect on plant fitness by fine tuning the plant defense response and returning non-beneficial DETs to a basic expression level (dashed green line).

6.4.4. De-priming could limit the impact of sequential stresses

It was shown that treatment with BTH increases the expression of the flg22 receptor *FLS2* as well as the closely related co-receptor *BAK1* (Tateda et al., 2014). An increased expression of *BAK1* was shown to have strongly reduced growth and extensive cell death (Domínguez-Ferreras et al., 2015). It might be possible that the subsequent flg22 exposure after BTH could fine tune the plant expression profile to prevent negative effects of BTH and to enhance the resistance of the plant. The gene ontology analysis of the category *O110* in our Arabidopsis comparisons revealed that most DETs belong to cellular and metabolic processes ((Suppl.) Figure 28). Indicating, that changes in the expression profile not only effects transcripts related with defense responses. In Figure 36 we propose a model of the effect of expressional de-priming. We hypothesize that the de-priming of a subset of transcripts could enhance plant fitness. The second treatment and the resulting de-priming of transcripts could prevent

the plants from negative effects of the priming treatment and/or fine tune the plant response to the more recent stress. It was shown with several examples that plants deal with stressful situations by inducing silencing mechanisms via antisense transcription and that endogenous siRNAs derived from a pair of sense and antisense transcript can enhance the tolerance to various stresses (Borsani et al., 2005; Chinnusamy et al., 2007; Jin et al., 2008; Sunkar, 2010; Khraiweh et al., 2012) The strong overrepresentation of antisense transcripts in the *Arabidopsis* sub-category *O110 up by BTH* is in line with our hypothesis, that plants induce de-priming in order to prevent negative or unnecessary effects of the priming stimulus. These antisense transcripts are up-regulated by BTH and down-regulated by the subsequent flg22 treatments. Therefore, it may be that enhanced antisense expression suppresses negative effects that the BTH treatment may have on the plant. With our work we describe a rather unusual expression profile caused by subsequent stresses in *Arabidopsis* and apple in which plants have an efficient way to memorize stresses by transcriptional priming, and that such priming can readily be erased (de-primed) by subsequent stresses. Since our observations were made in both apple and *Arabidopsis* suggests that priming and de-priming may be conserved in the plant kingdom. These results have implications on the application of priming compounds in the field, as plants are constantly subjected to stresses, which may affect the efficiency of such compounds.

6.5. Material and methods

6.5.1. Plant material

Plant material used was wild-type *Arabidopsis thaliana* L. Heynh cultivar 6 Columbia (Col-0) as well as *met1-3* (Saze et al., 2003) and *npr1-3* (Herr et al., 2005). All *Arabidopsis* plants were grown under long day condition (photoperiod of 16h light at 22°C/ 8h light at 22°C, with 60% relative humidity). Apple cv. Golden Delicious double haploid (GDDH13 described by (Lespinasse, 1998)) *in-vitro* grown plantlets were propagated on MS based medium with BA 0.25mg/L and IBA 0.1mg/l under short day conditions (photoperiod of 8h light at 22°C/ 16h dark at 21°C). Grafted apple plants (GDDH13) were grown in the greenhouse on a MM106 rootstock. Dormant buds were grafted in winter (February). After 10 days in a cold chamber

they were potted in 1L pots and grown in normal greenhouse conditions until they developed to about 30 nodes.

6.5.2. Quantification of the growth inhibiting effect of BTH

The growth inhibiting effect of BTH (Bion, Syngenta) was examined in grafted apple plants. Two months after grafting the plants were treated six times every three days with BTH (1mM) by applying the solution with a paintbrush on all leaves and the meristem. The total number of internodes was counted 3 weeks after the first treatment. The growth inhibiting effect of BTH on apple plantlets was observed by growing plantlets after propagation on MS based medium with 0.25mg/L BA, 0.1mg/L IBA and 1 μ M BTH or the control media without BTH for four weeks before the pictures were taken.

6.5.3. Transcriptomic analysis

Four Arabidopsis plants represent one biological replicate. Plants were treated with flg22 (QRLSTGSRINSAKDDAAGLQIA – obtained from Eurogentec SA (Angers, France)) or BTH (Bion) – obtained from Syngenta (Basel, Switzerland) and were applied by spraying with final concentrations of 1 μ M and 1mM, respectively. Leaves of 20 apple plantlets represent one biological replicate. Plantlets were treated by dipping the whole plantlet into filter sterilized flg22 (1 μ M) and BTH(1mM) solution and placed on fresh growth medium after every treatment. Microarray analysis was performed for Arabidopsis with the CATMA array ((V5) GPL21364)) and for apple with the ARIANE array (VXXX) Leaves of Arabidopsis and apple were collected from two independent biological replicates. The RNA was extracted using the NucleoSpin RNA plant extraction kit (Machery-Nagel Hoerd, France) according the manufacturer`s recommendations. For Arabidopsis samples the Message AmpII aRNA amplification kit (Ambion) (Thermo fisher scientific USA) and for apple samples the Low Input Quick Amp Labeling Kit, two-color (Agilent USA) was used for cDNA synthesis and hybridization. The hybridizations were performed on a NimbleGen Hybridization System 4 (mix mode B) at 42° overnight. Afterwards, the slides were washed, dried and scanned at 2 μ m resolution. NimbleGen MS 200 v1.2 software was used for microarray scans and the Agilent Feature Extraction 11.5 software was used to extract pair-data files from the scanned images. Statistical analysis was based on dye switch approach as described in (Depuydt et al.,

2009). All statistical analyses were performed using the R language (R Development Core Team, 2009); data were normalized with the lowess method, and differential expression analyses were performed using the *lmFit* function and the Bayes moderated t test using the package LIMMA (Smyth et al., 2005) from the Bioconductor project. Differentially expressed transcripts sorted by applying the binary code were selected for a p-value < 0.05.

6.5.4. Determination of gene expression by qPCR

4 plants per biological replicates were harvested, frozen and ground in liquid nitrogen. RNA from 100 mg of tissue was extracted using the NucleoSpin RNA plant extraction kit (Macherey-Nagel Hoerd, France). The DNase treatment was performed according to the manufacturer's recommendations. Per PCR reaction, complementary DNA was synthesized from 10 ng of total RNA extract with oligo(dT) primers using Moloney Murine Leukemia Virus Reverse Transcriptase according to the manufacturer's instructions (Promega). For quantitative real-time reverse transcription PCR (qPCR) in a 96-well format, the Chromo4™ System (Bio Rad) was used. Expression was normalized to *ACR12* (AT5G04740) using the qGene protocol (Muller et al., 2002). The Primers used are as followed: *ACR12* (AT5G04740) *acr12FW*: TTGTTGATGATCGCCGGAA, *acr12REV*: TGGAACAACGTCGTCATCGT; *AMY1* (At4G25000) *amy1FW*: AATACGGTTCAGAGCGGAA, *amy1REV*: CGGAAGTCCCACCTTCGAAA.

6.5.5. Measurement of reactive oxygen species

For ROS assays, leaf discs of three weeks old soil grown plants, were placed into each well of a white 96-well plate (Thermo Scientific, Waltham, USA) in 0.1 mL of water and kept in the dark overnight. For elicitation and ROS detection, horseradish peroxidase and luminol were added to a final concentration of 10 µg/mL and 100 µM, respectively. Luminescence was measured directly after addition of concentrated BTH solution (final concentration of 1mM) in a FLUOstar OPTIMA plate reader (BMG LABTECH, Offenburg, Germany).

6.5.6. Callose deposition

Leaf discs were vacuum infiltrated for 10 min with BTH (1mM) solution or water and kept floated in the solution for 24h. After leaf discs were fixed and destained in 1:3 acetic acid/ethanol until leaf tissue was completely transparent. After washing the leaf discs in 150 mM

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K₂HPO₄ for 30 min, the plant material was stained for 2 h in 150 mM K₂HPO₄ and 0.01% aniline blue. Callose depositions were quantified with a Leica DM1000 microscope equipped with a Qimaging Micropublisher 3.3 RTV camera using a DAPI filter.

6.6. Appendix: Does BTH/flg22 application influence defense responses and *Pseudomonas* resistance in a long-time memory fashion?

6.6.1. Introduction

Plants are under continuous stress but higher plants have evolved mechanisms that enables them to cope with environmental changes. This mechanism was established over a long period of evolution as sessile organisms (Tardif et al., 2007). The mechanism of priming enables plants to respond and adopt to recurring biotic and abiotic stresses. Primed plants display faster and/or stronger activation of various defense responses that are induced by a subsequent attack by pathogens, insects or in response to abiotic stress (Conrath et al., 2006). The activation of systemic acquired resistance (SAR) is key for the plant to overcome a pathogen infection. Pre-treatment with various stress-signaling molecules like JA, SA, or ABA as well as, pre-exposure to pathogens or herbivores resulted in an increased SAR (Goh et al., 2003; Jakab et al., 2005; Conrath et al., 2006; Bruce et al., 2007; Conrath, 2011; Rasmann et al., 2012; Slaughter et al., 2012; Bruce, 2014). SAR involves several steps and multiple pathways that lead to a strong defense response (Conrath, 2006). SAR induction and an increased resistance to a subsequent infection can be achieved by treatments with chemical stimuli. These compounds induce low-cost changes in the plant metabolism that include the accumulation of various metabolites (Mauch-Mani et al., 2017). One of these compounds is the SA analogue BTH. BTH is the main active compound of the commercialized plant defense inducing product Bion (Görlach et al., 1996). SA itself was shown to trigger several responses in plants. At a low dose it was reported to enhance the activation of the flg22- induced MITOGEN-ACTIVATED PROTEIN KINASE 3 (MPK3) and MPK6 (Yi et al., 2015). Also BTH induces the accumulation of inactive unphosphorylated MPK3 and MPK6 (Beckers et al., 2009). It was also shown that a activation of the SA pathway influence flg22 triggered short-term defense responses like the oxidative burst and the deposition of callose (Yi et al., 2014). Several studies also showed that defense inducing compounds can also change the plant protein levels during a priming phase (Balmer et al., 2015). Importantly, the protein levels of pattern recognition receptors and coreceptors increase after BTH treatments (Tateda et al., 2014). These findings suggest that the plants prepare their defensive system to enhance their sensitivity against potential attackers (Conrath et al., 2015). However, recently it was suggested that plants

might be also able to forget a previous stress (Crisp et al., 2016) (and this thesis). Priming could result in a sensitization in a way they response to false alarm signals (Mauch-Mani et al., 2017). The current hypothesis at the moment is that plants might forget previous stresses to avoid reduced development, yield and ultimate survival (Crisp et al., 2016). This hypothesis is supported by the results that transgenerational defense priming may be linked to the severity of the originally faced stress and that the initial stress is only memorized when applied repeatedly (Luna et al., 2012). Epigenetics widely contributes to the stress memory in plants (Bruce et al., 2007; Tsai et al., 2011; Ramírez-Carrasco et al., 2017). Epigenetics involves fast and reversible modifications. Therefore, it is likely that epigenetic mechanisms are involved in the removal of immune priming after certain stress-free generations or after longer time in the same generation in order to alleviate the potential costs.

6.6.2. Results and discussion

After our investigations on the inversion and de-priming of transcriptional memory by a subsequent flg22 treatment after BTH exposure, we decided to explore the effect of BTH and flg22 treatment combinations on the induction of short-term defense responses. The activation of MAP Kinases (MAPK) is one of the fastest defense responses (Pitzschke et al., 2009). Since it was shown that BTH treatment enhances the protein level of the receptor FLS2 and the coreceptors BAK1 (Tateda et al., 2014) as well as the level of unphosphorylated MAPK (Beckers et al., 2009) we studied the level of phosphorylated MAPK, induced by application of flg22, after a pre-treatment of BTH and/or flg22 (Figure 37). Arabidopsis plants were treated after 7dag with water (W) or BTH (B) and a second time after 10dag with water or flg22 (F). After a recovery period of 11 days plants were treated with flg22 in order to induce the phosphorylation of MAPK. Even though these preliminary results miss a loading control with coomassie blue staining it is clearly visible that no difference between the two times water treated plants the BTH/flg22 treatments can be observed (Figure 37 A). This might be due to the long recovery phase of 11 days after the last treatment. In line with this result is also our next observation. Plants pre-treated after 7dag and 10dag with water, BTH or flg22 (WW, WF, BW, BF) were treated after 21dag with flg22 to induce a ROS burst. All pre-treated plants induced a ROS burst with a comparable intensity (Figure 37 B). Finally, we decided to test the resistance of pre-treated plants to a *Pseudomonas* infection. It was previously reported that plants pre-treated repetitively with BTH show an increased resistance to

Pseudomonas infections (Kohler et al., 2002). Also, our results show that plants pre-treated three times with BTH after 7, 10 and 13dag show reduced bacteria growth 1dpi. However, all other pre-treatment combinations do not show any difference in bacterial growth (Figure **37 C**). These results miss a control of bacteria extraction after the infection (0dpi) as well as a second extraction at 2dpi. However, these results indicate that the inversion and de-priming in transcription might not reflect in short-term defense responses as well as resistance to *Pseudomonas* infection. The de-priming and return to a basic expression level pertain only a subset of transcripts. It is possible that these de-primed transcripts might be not critical for the induced resistance by BTH but might cause negative effects for the plant and loss of energy due to priming-related fitness costs. The subsequent stress after BTH could fine-tune the expression response of BTH pre-treated plants and could lead to a directed response to the current stress the plant is facing. However, the increased resistance against *Pseudomonas* after a three times BTH treatment is in line with the previously published results on BTH. This might due to the shorter recovery phase of 8 days after the last BTH treatment. The plant memory for BTH response might be limited and the induced resistance is increased after 8 days but not after 11 days. In conclusion we show, that Bion has an effect on resistance against *Pseudomonas* infection but that the inversion and de-priming of transcription shows no effect on the short-term defense responses MAPK activation and ROS bust as well as resistance. However, these results have a strong preliminary character and require additional experiments and replicates.

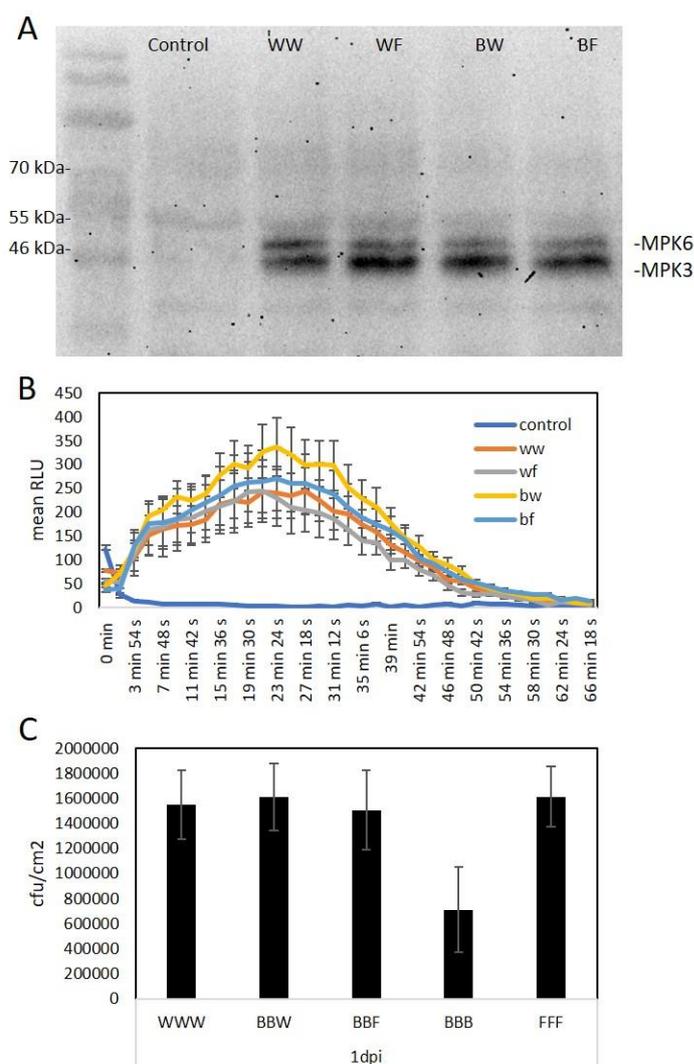


Figure 37: **Short-term defense and resistance to *Pseudomonas syringae* may not be influenced by de-priming.** **A)** Western blot against phosphor-p44/42 MAP kinase. Pre-treated plants after 7 and 10, were exposed to flg22 to induce the phosphorylation of MAPK. Pre-treated plants do not show an altered level of phosphorylated MAPK in comparison to the water treated control. **B)** ROS burst of pre-treated plants. Arabidopsis plants were pre-treated after 7 and 10 days with the indicated treatment and exposed to 1 μ M flg22 in order to induce the ROS burst. Pre-treatments do not have an effect on the ROS burst intensity. **C)** *Pseudomonas syringae* infection of pre-treated plants. Arabidopsis Plants were pre-treated by spraying after 7, 10 and 13 days with the indicated treatment. Plants three times exposed to BTH (1mM) show a reduced bacteria growth 1dpi (days post infection). None of the other applied pre-treatments caused an effect on plant resistance at 1dpi.

6.6.3. Material and methods

6.6.3.1. MAPK phosphorylation

Plants were pre-treated with a first treatment after 7dag (water (W) or BTH (1mM)(B)) and a second treatment after 10dag (water (W) or flg22 (F)). After a 11days recovery phase, all leaves of one plant were treated by floating for 15min on solution supplied with 1 μ M flg22 or without (control). After 15min leaves were shock frozen and grounded to fine powder before adding 80 μ l extraction buffer (0.35M Tris-HCl pH6.8, 30% glycerol, 10% SDS, 0.6M DTT, 0.012% bromphenol blue). After boiling for 5min, 15 μ l of the protein extract was separated by electrophoresis in 12% SDS- polyacrylamide gel and electrophoretically transferred to a polyvinylidene fluoride membrane according to the manufactural instruction (Milipore). For exposure, monoclonal primary antibodies against phosphor-p44/42 MAP kinase (cell Signalling Technologies) and alkaline phosphatase-conjugated anti-rabbit secondary antibody (Sigma Aldrich). Signal was detected using CDPstar (Roche).

6.6.3.2. Plant inoculation with *Pseudomonas syringae* pv. *Tomato* (pst. DC3000)

Plants were pre-treated with the indicated treatments after 7, 10 and 13dag with the indicated treatment combination and infected after 21dag. Eight leaves of one plant per pre-treatment were infiltrated with bacterial suspensions of at a concentration of 10⁵ colony forming units (cfu/ml) (OD600 of 0.02) in sterile 10mM MgCl (also used for mock-inoculation) using a needleless syringe. Plants were maintained at high humidity. Samples were taken using a cork-borer (d=8mm) to cut one leaf discs per infected leaf. Leaf discs were ground in 10mM MgCl, diluted to the indicated concentration and plated as droplets of 10 μ l on YEB plates with the appropriate selection. Plates were incubated at 28°C and colonies counted 1dap.

7. General Discussion

7.1. The discovery of the SCOOP peptide family

Considering the complexity of the plant immune system and the tradeoff between defense responses and plant development it is reasonable for plants to merge these two fundamental mechanisms. The expression of phytochemicals is a plant achievement that facilitates the induction of defense responses upon an infection and wounding as well as the regulation of plant development. The latest trend in small endogenous peptide research is the use of bioinformatic tools. By using the continuous advances in genomics and transcriptomics technologies already several small peptides have been identified, among them well known and increasingly well-characterized peptides such as CLE, EPIDERMAL PATTERNING FACTOR (EPF1), C-terminally encoded peptide 1 (CEP1) and ROOT MERISTEM GROWTH FACTOR (RGF) (Matsubayashi, 2014). Finally, our research adds another small peptide family onto this list. The bioinformatic prediction of the SCOOP family was only possible because of the advantage of using bioinformatics which can overcome the barrier posed by strong gene redundancy and the low abundance of peptides in the plant tissue (Matsubayashi, 2014). By finding analogues of the SCOOP peptide family in 74 Brassicaceae species and an alignment of the PROSCOOP sequences, we show that it is possible to precisely identify potentially active small peptide sequences. However, by using a bioinformatic approach we predict peptides that might not reflect real *in planta* peptides. The research on small peptides resulted in the identification of several peptides with unknown mature peptide structure. For instance, CLE40 was found in a comparable approach than the SCOOP family. By an *in silico* screen of CLV3 homologues CLE40 was found to control stem cell fate in the root meristem (Stahl et al., 2009). Other examples of small peptides without fully characterized mature peptide aa composition are CLE8, CLE45 and IDA. Even though, the full mature peptide has not yet been identified, the research on these peptides resulted in the identification of various interesting regulatory functions (Matsubayashi, 2014). The applied bioinformatic approach lead not only to the prediction of several active peptides but also predicted in which tissue the peptide precursors are expressed and helped us to predict functional partners. The use of the CATMA

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transcriptome database (Crowe et al., 2003; Sclep et al., 2007; Gagnot et al., 2008) is an excellent tool to identify previously unknown proteins. The microarray produced in frame of the CATMA project contains 677 gene specific tags that map in intergenic regions according to an alternative Arabidopsis genome annotation. By investigating the regions of these tags, it is possible to reveal possible under-predicted genes in Arabidopsis. Especially small peptides could be found by this method (Aubourg et al., 2007).

7.2. Possible posttranscriptional modifications of the SCOOP family

Next to the identification of *in planta* peptides, the discovery of posttranscriptional modification and secretion processes of small peptides bear a great challenge for researchers. Posttranscriptional modifications of small endogenous peptides are known to modulate the physiochemical properties, changes the net charge, alters the hydrophilicity and/or the conformation. These modulations therefore change the binding and specificity of the peptides and their receptors (Matsubayashi, 2014). Three such modifications have been identified for peptides up to now: tyrosine sulfation, proline hydroxylation and hydroxyproline arabinosylation (Matsubayashi, 2011). Upon them Proline hydroxylation is identified in almost all posttranscriptional modified small peptides. Only PSK does not carry this modification because it has no proline residues. Interestingly, out of the 4 inactive SCOOP peptides (SCOOP3, 8, 10, 11) the peptides SCOOP8 and SCOOP11 do not harbor any proline residue. Out of the active peptides only SCOOP5 does not contain a proline residue. However, the activity of SCOOP5 might be promoted by a tyrosine sulfation. Overall only SCOOP5 and SCOOP13 contain a tyrosine residue. Tyrosine sulfation is a modification that indicates a peptide which is synthesized through the secretory pathway (Moore, 2003). Three other tyrosine-sulfated peptides have been identified in plants namely, PSK, PSY and RGF (Matsubayashi and Sakagami, 1997; Amano et al., 2007; Matsuzaki et al., 2010). Another open question in regard to our SCOOP peptide discovery is the possibility of a posttranslational proteolytic processing of the precursor protein. The identification of the precursor cleavage site of plant peptides is challenging. There is no typical amino acid motif in plants which is directly adjacent to mature peptide domains. Moreover, the nature of the cleavage mechanism is not always clear in plants. In animals, small peptides are cleaved on the C-

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terminus of paired basic amino acids, such as lysine-lysine, lysine-arginine or arginine-arginine. The CLV3 peptide in plants is cleaved on the N-terminus at a single arginine residue of its precursor protein (Ni and Clark, 2006). Another example is CLE36 which is cleaved between a methionine and a serine residue is located two residues upstream of the mature peptide domain (Djordjevic et al., 2011). Therefore, the processing site does not define the boundary of the mature peptide domain because, next to the proteolytic cleavage, peptides undergo proteolytic trimming. In summary, the posttranslational modifications could protect the peptide from exoprotease digestion. The conserved active motif of the SCOOP peptides is not located at the extreme C-terminus of the precursor proteins which could mean that SCOOP peptides have to undergo two proteolytic cleavages. However, the majority of SCOOP peptides harbor a second conserved motif which is located closer to the N-terminus than the SCOOP motif, the second motif might reflect a possible cleavage site.

7.3. The identification of the SCOOP receptor(s) – the next big challenge

The next challenge which future research on SCOOP peptides will face is the identification of the SCOOP receptor. Several promising methods have been developed in order to identify pattern recognition receptors. The most efficient approaches are forward genetic mutant screens. The two tools which are often applied are the use of natural variations and the use of a mutagenized population. The most prominent example of a receptor that was identified by using an EMS mutagenized population of Arabidopsis is the flg22 receptor FLS2 (Gomez-Gomez and Boller, 2000). Treatments with the active SCOOP peptides causes a strong inhibition of seedling growth. This phenotype could be used to identify the receptor or other functional partner in the SCOOP perception by screening EMS mutagenized seeds for insensitivity to SCOOP peptides. The seedling growth inhibition assay was also the assay of choice for the identification of the FLS2 receptor. An alternative method to identify peptide receptors are biochemical approaches. One example is the discovery of the PEPR1 receptor which was identified using radiolabeled *AtPep1* peptide, interacted with suspension-cultured Arabidopsis, followed by binding studies (Yamaguchi et al., 2006). Also, possible and likely to be successful for the SCOOP peptides is the use of a co-receptor as a molecular bait. It was for example shown that it is also possible to identify FLS2 after flg22 treatment and

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immunoprecipitation of BAK1 (Schulze et al., 2010). We could show that perception of at least SCOOP6, 7 and 12 is dependent on BAK1. Moreover, we show that the *bak1-4* mutant is almost completely impaired in SCOOP12 perception. A BAK1 immunoprecipitation could help to identify the SCOOP peptide receptor. However, BAK1 has been shown to serve as co-receptor to a high number of PRR receptors (Chinchilla et al., 2009). An immunoprecipitation might thus lead to many false positive receptor candidates. Another challenge for the discovery of the SCOOP peptides might be the possibility that not all SCOOP peptides are perceived by the same receptor. The SCOOP peptide motif is very diverse in comparison to other small peptides. It could be possible that different SCOOP peptides might be perceived by different receptors. This was also shown for the AtPeps. While AtPep 1-6 are mainly perceived by PEPR1, AtPep1 and 2 are perceived by PEPR2 (Yamaguchi et al., 2010; Bartels and Boller, 2015; Lori et al., 2015). It was recently shown that the complexity of receptor and coreceptor interactions can be very high. The investigation of 40,000 potential extracellular domains and their interactions revealed a regulatory network consisting of 567 interactions (Smakowska-Luzan et al., 2018). Interestingly, the regulatory network confirms BAK1 as the most important and interconnected node while the authors identified a previously unknown LRR-RK (APEX). The double mutant of BAK1 and APEX is strongly impaired in plant development. The authors also confirmed APEX as an interaction partner of PEPR1 and PEPR2 in mediating AtPep2 induced ROS burst (Smakowska-Luzan et al., 2018). This indicates that APEX might also be involved in the perception of the SCOOP peptides. However, our predictions of PROSCOOP12 coregulated genes could not confirm APEX to be coregulated.

7.4. Phyto cytokines – peptides between defense and development

The strongly reduced growth phenotype of *apex bak1-5* is another observation demonstrating the importance of small endogenous peptides and their receptors in plant development. With that respect, the SCOOP family is not an exception, since we show that the SCOOP peptides are involved in root development. We conclude that the SCOOP family is a new phyto cytokine. The term phyto cytokines was just recently defined as secondary endogenous peptides which require a processing step and are passively or actively released. The SCOOP family fulfills all properties of a phyto cytokine and is comparable with other members of this

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classification. The *AtPIP* peptide family that was described in 2014 is probably the closest functional homologue of the SCOOP family (Hou et al., 2014). Similar to the PROSCOOP proteins the precursor of *AtPIPs* (*prePIPs*) harbor an N-terminal secretion signal and a conserved C-terminal peptide motive whereas the synthetic peptide PIP1 induces defense responses in *Arabidopsis*. Like SCOOP12, the PIP1 peptide induces the expression its own precursor. Both peptide families interact with *AtPep* signaling by inducing the expression of PROPEPs. PIP1 induces the expression of PROPEP1 and SCOOP12 the expression of PROPEP2. PIP1 was also shown to amplify FLS2 signaling and therefore bridges the perception of MAMPs with the action of phyto cytokines (Hou et al., 2014). The three peptide families *AtPIP*, *AtPep* and SCOOP seems to be tightly linked in their function as amplifier of defense. Even though, all three peptides have different receptors, BAK1 is involved in the perception of peptides of these families (Chinchilla et al., 2009; Hou et al., 2014). Moreover, the downstream signal after perception of members of these three peptides families seems to be identical. The expression of FRK1 is enhanced after treatments with peptide members of the three families (Flury et al., 2013; Hou et al., 2014). It was proposed that receptors operate in unified regulatory networks (Smakowska-Luzan et al., 2018). This thought leads to the idea that the corresponding ligands might also act in larger networks. Different small peptides as well as their precursors have very similar effects on plant defense responses and plant development. It might be, that the peptides cooperate in the action of defense stimulation while the precursors have antagonistic effect in root (or whole plant) development. In line of this idea the observation that overexpression of *AtPROPEP1* results in a bushy root phenotype and the most *AtPROPEPs* and both *AtPEPRs* were found to be exclusively expressed in roots is an interesting novel observation (Huffaker et al., 2006; Ferrari et al., 2013). Overexpression of *prePIP1* *prePIP2* reduces root growth and knocking out these genes leads to an increased root growth. This indicates that PROSCOOP12 and *prePIP1* have comparable functions in root development which might be antagonistic regulated by PROPEP1. A high number of small endogenous peptide families are found to have a function in root development (Delay et al., 2013). An orchestration of peptides in order to control various biological functions was shown in the case of the CLE peptides. CLE peptide signaling integrated with phytohormone signaling controls developmental processes in various tissues. Especially auxin signaling conjugates with different CLE peptides in regulating vascular patterning (Wang et al., 2015). The fact that *AtPep*, *AtPIP* and SCOOP are expressed in the same tissues and the growing evidence that the

Pep-PROPEP-PEPR system is involved in developmental processes (Huffaker et al., 2006; Gully et al., 2015) could indicate an orchestration of these families similar to the CLE peptides.

7.5. DAMPs and synthetic elicitors as plant defense stimulating compounds

The idea of using classical DAMPs as vaccines to promote the plant defense is also adopted by agricultural companies and the first products based on plant extracts are already available on the market (Quintana-Rodriguez et al., 2018). This raises the question of possible advantages of these plant extract products compared to synthetic elicitors. One advantage of crude plant extracts is that is easily and rapidly prepared and has therefore a low economic cost. This could make them an option for low-income farmers worldwide. However, the exact concentration of the active compound in the plant extract remains unknown. Plant extracts contain a high diversity of DAMPs and therefore they are expected to trigger a more diverse set of resistance responses compared to an individual DAMP. Moreover, plant extracts could have direct pathogen repellent or antimicrobial effects (Quintana-Rodriguez et al., 2018). However, the possible effects of plant extracts treatments are not completely clear. It might be possible that plant extracts contain pathogens or plant viruses that bear the risk of infection on the treated plant. Another disadvantage is the limited stability of the containing DAMPs that probably would limit the applicability on the field. Overall, the use of synthetic defense-promoting compounds is still the more adapted tool in crop protection. An intersection of DAMPs and synthetic elicitors as treatment is given by two studies that showed that treatment with BTH leads to a release of volatile organic compounds (VOCs), which can be classified as DAMPs, from lima beans. These VOCs elicited resistance against a pathogen in neighboring lime bean plants (Yi et al., 2009; Heil and Adame-Álvarez, 2010).

The most important advantage of synthetic elicitors is the broad activation of defense responses including the accumulation of salicylic acid. SA is the most important hormone involved in systemic acquired resistance, which is induced in distal non-treated parts of the plant. SAR promotes resistance against a broad spectrum of pathogens (Henry et al., 2013; Faize and Faize, 2018). The effect of SA in plant defense is therefore of great magnitude. However, SA is not being directly used as defense promoting compound. Instead, the

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compounds of choice are SA analogues. SA itself gets rapidly glycosylated or methylated which reduces its activity (Vlot et al., 2009; Yamasaki et al., 2013). Moreover, several examples showed a phytotoxic action of SA and, when applied in higher concentrations, an inhibition of growth and an induction of strong defense responses (Heil and Baldwin, 2002). The methylation of SA generates MeSA, which is a possible mobile molecule active in SAR. However, only a few commercialized functional analogues of SA carry a methyl modification. Among them is BTH, which might enable the induction of SAR in various plants and against a wide range of pathogens. Even though, BTH is a functional analogue of SA, it just mimics a subset of SA functions and is interfering with the receptors and triggers similar transcriptional and physiological responses. However, SA analogues do not directly interfere with SA targets (Bektas and Eulgem, 2014). Moreover, farmers face several problems by using SA analogues. This includes a very incomplete disease reduction and the necessity of frequent application. Moreover, a recent study showed that the formulated BTH (Bion) photolyze quickly on detached apple leaves with a half-life of only 2,8h (Sleiman et al., 2017). This result might explain the requirement of repetitive applications. The problem of a reduced plant fitness, as it was previously described for SA, is still an issue for treatments with SA analogues (Canet et al., 2010; Faize and Faize, 2018) (and this work). This raises the question if SA analogues and especially BTH are really the optimal choice as defense activators. Also, our research shows that BTH application causes a growth inhibition effect in Arabidopsis and apple. The negative effects of SA analogues and the largely unknown molecular mechanism made it necessary to screen for new compounds in defense priming. One research group developed a high-throughput screening method to identify compounds which potentiate pathogen-activated cell death in Arabidopsis cell cultures. This group found and characterized in a series of publications five compounds that prime immune response (Noutoshi et al., 2012b; Noutoshi et al., 2012c; Noutoshi et al., 2012a). The group screened 10.000 molecules and only three induced a priming effect without causing cell death. The newly identified molecules are called imprimatins. Interestingly, two of these molecules achieve their action by inhibiting the SA glucosyltransferase (SAGT) which then allows the accumulation of endogenous SA. (Noutoshi et al., 2012a). The idea of overcoming the tradeoff between defense and growth by the development of defense-priming compounds which do not directly interfere with SA receptors and pathways, but which cause changes in the SA metabolism, transport and perception might be the future of defense priming compounds. Another possible strategy to

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identify new priming compounds was described recently. Kuai and colleagues proposed that research should focus on the development of “Just in time” immunomodulating compounds. These compounds should be chemicals that boost immunity on demand and only when needed (Kuai et al., 2017). They propose to develop an NPR1 agonist, since NPR1 is a key factor in SA signaling.

In fact, the action of NPR1 and the action on defense priming by SA analogues is mostly in focus of research while the problem of the defense and development tradeoff remains to be investigated in more detail. One of the key factors in the balance of defense and development are the target of rapamycin (TOR) kinase and raptor (regulatory-associated protein of mTOR). A recent study on rice showed that plants with reduced TOR signaling display enhanced disease resistance against bacterial and fungal pathogens. TOR was found to antagonize the action of SA and JA. Moreover, the authors could show that silencing of raptor primes SA and JA dependent gene expression in response to BTH (De Vleeschauwer et al., 2018). In human cancer research a lot of strong and weak inhibitors of mTOR are in focus of research. Moreover, numerous anti-cancer drugs are plant natural compounds. Just one example out of many is resveratrol, a phytoalexin found in grapes (Zhang et al., 2012a). It would be interesting to investigate the effect of a combined treatment of a salicylic acid analogue such as BTH and a TOR inhibitor. A combined treatment of plant extracts containing natural TOR inhibitors and BTH could lead to stronger plant protection activity.

7.6. Is a plant able to forget?

One question that this thesis raised is whether plants are able to “forget” a previous stress or treatment. The quotation by Nietzsche *“Without forgetting it is quite impossible to live at all”* fits very well to the life of a plant. Plants are not able to escape a stressful situation and face many different stresses in short periods of time. The concept of stress memory is in the focus of research however the resetting of memory is at least of the same importance. A time when the concept of resetting versus consolidation of memory is essential is during the period of stress recovery. A stress recovery phase is defined to be a period of time following a stress until a new homeostasis is attained (Crisp et al., 2016). A new higher homeostasis can be

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reached due to the priming and memory of a plant while in other cases the post-stress homeostasis resembles the prestress state. This is the case if the stress is brief and a memory of the stress would be a maladaptation for the plant. It might also be the case that a stress is prolonged but there is still a difference between pre- and post-stress state. This might be due to a progression of the plant to a new developmental stage (Crisp et al., 2016). The possibility of different developmental stages we avoid in our research by using *in-vitro* apple plantlets. In contrast to apple trees these plants keep their vegetative developmental stage and do not multiply sexually. The concept of stress recovery and therefore a reset of memory or transcription is especially important when the initial stress could have a long duration and the stressful situation could vanish rapidly which then requires a fast adaptation. This is the case for drought stress. Therefore, several investigations have analyzed rehydration responses during drought stress recovery. One study showed that drought stressed *Medicago truncatula* undergo a global reprogramming of transcription and metabolism during 14-days of drought and re-watering. Moreover, 90% of the drought-responsive genes react oppositely to the addition of water (Zhang et al., 2014). Thus, a stress and a subsequent stress release leads to an inversion of transcription. Another interesting publication showed that not only a stress release but also a similar repeated stress can lead to a transcriptional inversion. Liu and colleagues showed that a subset of dehydration stress response genes respond to an initial drought stress and return to a basic expression level during watered recovery but do not respond to a subsequent stress exposure (Liu et al., 2014). Interestingly, with our results we show that a subset of genes returns to a basic expression level by applying a second biotic stress in form of flg22. Even though BTH and flg22 have the capacity to induce or to prime defense responses, a subsequent treatment of flg22 after BTH inverts the expression of a subset of transcripts. An inversion of expression by a comparable treatment was, by our knowledge, not described before in the literature. A possible explanation could be that BTH application is more stressful for the plant than the subsequent flg22 treatment. The flg22 treatment condition is therefore the more favorable condition. Under natural growth conditions plants may sense flg22 continually and the “flg22-stress” condition could be considered as a baseline stress. On the other hand, BTH is not seen by the plant in an all-day fashion which therefore could be considered as a stress trigger. However, with our research we investigate the transcript expression profile several days after the last treatment. Thus, we can propose two mechanisms of flg22-induced de-priming of BTH induced transcripts. First

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it is possible that flg22 treatment leads to an accelerated returning to the basic expression level during the last recovery phase before sampling. The second possibility is that flg22 leads to an immediate de-priming of BTH-induced transcription expression. To further investigate these two possibilities, it would be important to follow the expression profile of the de-priming marker gene *AMY1* that was identified in this thesis directly after the treatments. It would also be possible to follow the expression of *AMY1* by fusion of the promotor with a small and rapidly degrading reported gene such as the yellow fluorescence protein (YFP) of luciferase.

7.7. Molecular mechanism of de-priming

Investigations of de-priming velocity might give us an indication of the molecular mechanism behind our observations. As described in the introduction, stress priming can be established on the transcriptional level or by epigenetic changes. Also, the returning to a basic expression level could be regulated at the transcriptional or epigenetic level. It was shown that the mRNA half-life can have a range of 0.2 to 24 hours in Arabidopsis (Narsai et al., 2007). However, it was also shown that stress-responsive mRNAs can be stabilized by RNA binding proteins (Frei dit Frey et al., 2010). It would be interesting to investigate the role of the RNA binding protein such as Tudor-SN in response to the subsequent flg22 treatment after BTH. Tudor SN is one of the best studied RNA binding protein (Frei dit Frey et al., 2010). With our research we show that among the differently expressed transcripts that show the de-priming pattern, an overrepresentation of antisense transcripts can be found. It was reported that naturally occurring antisense RNAs (natsiRNAs) have the potential to affect mRNA expression during stress in an inverse relationship by silencing sense transcripts, as several reports have reported (Zhang et al., 2012b; Wang et al., 2017). Although, natsiRNAs have the potential to be key players in the inversion of transcriptional memory their role in this process remains to be explored. It would be interesting to identify natsiRNAs accumulation after BTH and BTH with subsequent flg22 treatment by northern blot and to find the target genes. The “forgetting” of the transcriptional memory could be also be due to a change at the transcriptional level in from of removing template RNA molecules that could be used by the posttranscriptional gene silencing (PTGS) or RdDM pathways (Crisp et al., 2016). Also, our results link de-priming with

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the RdDM pathway. In the *nRPD1-3* mutant background the expression of AMY1 is strongly reduced. This could be an indication for the existence of a negative regulator for AMY1 which is epigenetically silenced in the wildtype situation and that shows an enhanced expression in hypomethylated mutant background. AMY1 is from an “epigenetic point of view” a gene which is at first sight not under epigenetic control. Our investigations showed no hypo or hypermethylation at the AMY1 gene in the wildtype background (data not shown). However, since AMY1 is nearly not expressed in the *met1-3* mutant background, we can assume a role of DNA methylation, and probably in CG context, in the regulation of AMY1. It would be necessary to repeat the microarrays in the *met1-3* and *nRPD1-3* mutant background to investigate the global gene expression profile after BTH and BTH with subsequent flg22 treatment. Moreover, it would be interesting to investigate the global methylation level by bisulfite sequencing of plants treated with BTH and BTH/flg22. With this experiment we would be able to identify differently methylated regions (DMRs) involved in de-priming of expression.

One sort of “forgetting” of stress-induced priming is often showed by a change in generations. Not many publications showed a transgenerational transmission of acquired traits (over one stress-free generation). An interesting publication identified two chromatin regulators to be impaired in the resetting of stress induced loss of epigenetic silencing. *DDM1* and *MOM1* have been identified to be factors preventing transgenerational inheritance (Iwasaki and Paszkowski, 2014). The activity of *MOM1* is linked to transcriptional gene silencing however, *MOM1* action was also shown to contribute to the accumulation of small interfering RNAs in order to control gene silencing by the RdDM pathway (Yokthongwattana et al., 2010). It would be interesting to repeat the microarray setup (or to follow the expression of *AMY1*) in *Arabidopsis* using the *mom1 ddm1* double mutant in order to explore the role of these chromatin regulators in memory resetting in addition to their role in preventing transgenerational epigenetic memory. Interestingly the authors who identified *MOM1* and *DDM1* involvement in resetting transgenerational memory proposed that the prevention of transgenerational memory extends far beyond the activity of *MOM1* and *DDM1*. They found around 3000 loci which are activated under stress conditions but still 340 transcripts remain transgenerationally active in the *mom1 ddm1* double mutant (Iwasaki and Paszkowski, 2014).

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This indicates that also the mechanism of de-priming might be under complex epigenetic and/or transcriptional regulation.

7.8. Conclusion

With this thesis I show a wide range of different aspects of plant immunity and development. Due to the two projects I followed during my thesis I can provide results here describing the discovery and characterization of a new small endogenous peptide family called SCOOP and I describe an unusual memory response by a subsequent flg22 exposure after treatments with the defense priming compound BTH.

In the first part of my thesis I show that one member of the SCOOP family, namely SCOOP12 can be classified as a new posttranslational modified phyto cytokine with plays roles in plant defense responses and root development. Although, the SCOOP peptide family shares many features with other described small endogenous peptide families like AtPep and AtPIP, the precursor of the SCOOP peptides, PROSCOOPs have a very diverse C-terminus, which indicates a great variety of the processed peptides and therefore a broad spectrum of functions. Further, I conclude that the SCOOP peptide family contains various members with the capacity to induce defense responses and with strong effects on root development. However, the exact mode of action of the SCOOP peptides on the root defense and development remains for the moment unclear. Moreover, I show that the members of the SCOOP peptide family, similar to other peptide families, is perceived by members of the SERK receptor family. The perception of SCOOP12 is highly depended on BAK1. In conclusion, my research on the SCOOP peptide family could be the onset and initiation for the discovery of many additional functions of the SCOOP peptide family in Arabidopsis and other Brassicaceae species members.

In the second part of my thesis I conclude that the priming state of BTH induced transcriptional memory can be forced to return to a basic expression by a subsequent flg22 treatment. I show that this effect is not only present in Arabidopsis but also in the economically important plant apple. The identification of a de-priming marker gene lead to

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first evidence that the global methylation could be crucial for de-priming of BTH-induced differently expressed transcripts.

In conclusion, my thesis investigates effects on plant transcription, development and defense by endogenous (the SCOOP peptide family), exogenous (flg22) and synthetic (BTH) plant elicitors. My thesis shows how diverse the function of these elicitors can be and how the plant defense system as well as the triggers of plant defense can cause tremendous effects on plant development and memory. A coarser overview over the main findings on Arabidopsis and apple are summarized in Figure 38.

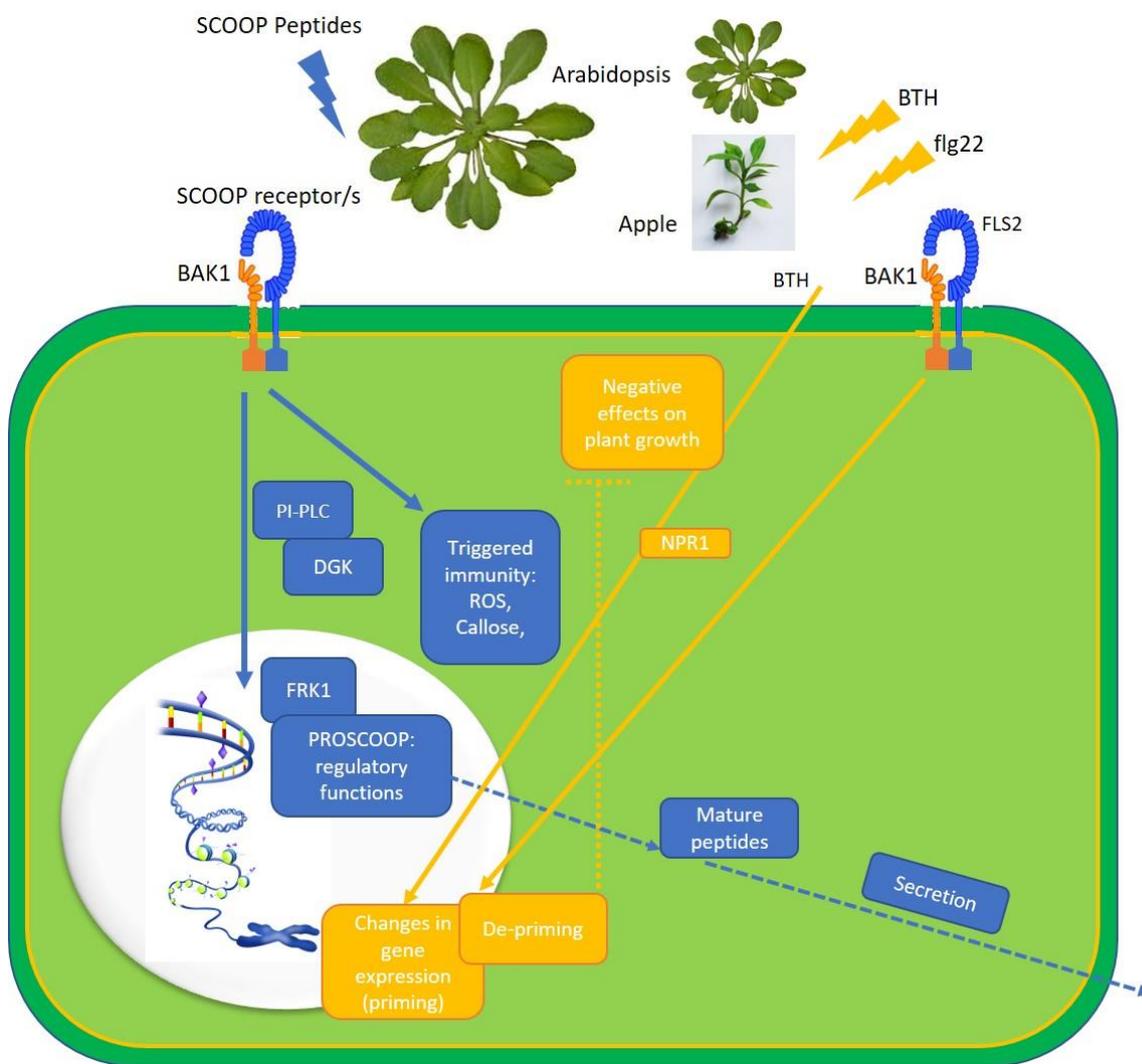


Figure 38: Summary of the main findings. My thesis shows results on mainly on two plant species namely Arabidopsis and apple. The SCOOP peptide family was found to be restricted to Brassicaceae family while the de-priming expression profile was observed in Arabidopsis and apple. Results correlated with my work on the SCOOP peptide family are indicated in blue and results on the de-priming expression profile in orange. Dotted lines represent putative interactions.

7.9. Outlook

Despite, our investigations into the actions, processing and perception of the SCOOP family members, many open questions remain unanswered.

In the general discussion I propose several methods with the goal to identify the SCOOP receptor or the SCOOP receptors as well as the identification of the mature SCOOP peptides. These experiments should be carried out to further characterize the SCOOP peptide family. It would also be important to expand our knowledge about other SCOOP precursor proteins apart from PROSCOOP12. The bioinformatic prediction of the SCOOP family expression profile revealed that PROSCOOP12 displays rather an exception in comparison to the other SCOOP peptides. This could indicate a different role in developmental processes, since also not all SCOOP family members induce defense responses. It would be interesting to confirm the bioinformatic prediction by fusion of the PROSCOOP genes with a GUS reporter gene. Besides the localization of the PROSCOOP genes it would be interesting to generate knock out and overexpression mutants of the PROSCOOP genes. These experiments would be especially important because mutants of the precursor proteins of small endogenous peptides did not receive a lot of attention so far (Trivilin et al., 2014). The PROPEPs were assumed to act redundantly and therefore the receptor mutant was used to indirectly study the lack of PROPEPs and PEPs (Bartels and Boller, 2015). However, we have a clear indication, by bioinformatic prediction, that members of the SCOOP family are involved in different cellular processes. Further, it could be possible to use the CRISPR-Cas9 approach to knock out several PROSCOOP members at the same time, due to the conserved motif. The research on SCOOP peptide family could be facilitated by specific antibodies targeting the PROSCOOP proteins. By in situ hybridization we could generate a tissue specific map of PROSCOOP presence. Further, due to the increasing evidence that phytochemicals are involved in developmental processes, it would be good to investigate possible interactions within different families like *AtPep*, *AtPIP* and SCOOP.

The results presented here show that transcriptional regulation is a very plastic mechanism. Since the effect of BTH and the de-priming of a subsequent flg22 exposure affects global expression, it would be important to investigate global effects on DNA-methylation of BTH

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and BTH/flg22. Since we have hints that the DNA methylation state is key to de-priming we should perform a bisulfide sequencing in order to find differentially methylated regions that might help us to identify factors involved in de-priming. Further, it would be important to not only focus on de-priming but also on mechanisms involved in the memory of BTH treatments. Not many stresses have been shown to have a transgenerational effect. However, it was shown that next generational SAR requires the activity of NPR1, indicating that SA or SA signaling can lead to transgenerational resistance in *Arabidopsis* (Luna et al., 2012). This raises the question if this transgenerational inheritance can be also observed in apple. Apple plants are frequently treated with salicylic acid analogues like BTH. This question could be addressed by comparing methylomes of untreated plants with BTH treated trees. Priming is widely associated with histone modifications. Several histone modifications are connected with defense priming namely H3K4me3, H3K4me2, H3K9ac, H4K5ac, H4K8ac and H4K12ac. Especially, H3K4me3 is considered as primary chromatin mark of stress memory (Conrath et al., 2015). Up to now, promoter regions of three defense-associated genes have been shown to be under epigenetic control upon BTH treatment (Jaskiewicz et al., 2011). It would be interesting to apply ChIP experiments with subsequent sequencing with antibodies of the mentioned histone marks. These experiments would open a new door for the identification of memory genes under epigenetic control upon BTH and BTH/flg22 treatment. Once more, factors are identified showing different histone mark distribution it would be interesting to test other defense activating compounds apart from BTH, like BABA, in order to find reliable defense priming genes. Moreover, by using flg22 we induce a PTI response, it would be interesting to trigger an additional ETI response by using bacteria like *Pseudomonas* on *Arabidopsis* and *Erwinia amylovora* on apple plants. We assume that the flg22-induced de-priming expression profile might counteract negative effects of BTH. This hypothesis needs to be experimental tested. We show that BTH causes an inhibition of growth that could be explained by a tradeoff between growth and defense. It might be that the subsequent flg22 treatment causes a shift of resources. Interesting would be to apply a metabolite profiling by liquid chromatography coupled with mass spectrometry of BTH and BTH/flg22 treated plants.

Despite these open questions, the findings presented in this thesis could have an impact on the future research on small endogenous peptides and the agricultural use of plant defense inducing compounds. With the SCOOP peptides we add now a new peptide family to the class

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of phyto cytokines. This discovery might pave the way to new findings regarding the involvement of small endogenous peptides in defense and development. The observed de-priming memory response in Arabidopsis and apple could appeal the discussion of forgetting mechanisms in plants.

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10. Appendix: Scientific publication (Gully et.al., 2018 Journal of experimental botany)

The SCOOP12 peptide regulates defense response and root elongation in *Arabidopsis thaliana*.

Kay Gully^{1‡}, Sandra Pelletier^{1‡}, Marie-Charlotte Guillou¹, Marina Ferrand², Sophie Aligon¹, Igor Pokotylo³, Adrien Perrin¹, Emilie Vergne¹, Mathilde Fagard², Eric Ruelland³, Philippe Grappin¹, Etienne Bucher¹, Jean-Pierre Renou^{1*}, Sébastien Aubourg^{1*}.

¹ IRHS (Institut de Recherche en Horticulture et Semences), UMR 1345, INRA, Agrocampus-Ouest, Université d'Angers, SFR 4207 QuaSaV, Beaucouzé F-49071, France.

kay.gully@inra.fr, sandra.pelletier@inra.fr, marie-charlotte.guillou@inra.fr,
sophie.aligon@agrocampus-ouest.fr, adrien.perrin@inra.fr, emilie.vergne@inra.fr,
philippe.grappin@agrocampus-ouest.fr, etienne.bucher@inra.fr, jean-pierre.renou@inra.fr,
sebastien.aubourg@inra.fr

² Institut Jean-Pierre Bourgin, INRA, AgroParistech, CNRS, Université Paris-Saclay, RD10, Versailles F-78026, France.

marina.ferrand@inra.fr, mathilde.fagard@inra.fr

³ iEES-Paris (Interaction Plantes-Environnement Institut d'Ecologie et des Sciences de l'Environnement de Paris), UMR CNRS 7618, Université Paris Est Créteil, 61 avenue du général de Gaulle, Créteil F-94000, France.

pokotylo_i@ukr.net, eric.ruelland@u-pec.fr

‡ These authors contributed equally to this work

* Co-corresponding authors

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The SCOOP12 peptide regulates defense response and root elongation in *Arabidopsis thaliana*.

Running title

Characterization of a new *Arabidopsis* phytoytokine.

Highlight

A secreted peptide, member of a Brassicaceae specific gene family, acts on pathogen defense response and root development through phospholipid pathway and ROS regulation.

Abstract

Small secreted peptides are important actors in plant development and stress response. Using a targeted *in silico* approach, we identified a family of 14 *Arabidopsis* genes encoding precursors of serine rich endogenous peptides (PROSCOOP). Transcriptomic analyses revealed that one member of this family, *PROSCOOP12*, is involved in processes linked to biotic and oxidative stress as well as root growth. Plants defective in this gene were less susceptible to *Erwinia amylovora* infection and showed an enhanced root growth phenotype. In *PROSCOOP12* we identified a conserved motif potentially coding for a small secreted peptide. Exogenous application of synthetic predicted SCOOP12 peptide induces various defense responses in *Arabidopsis*. Our findings show that SCOOP12 has numerous properties of phytoytokines, activates phospholipid signaling pathway, regulates ROS response, and is perceived in a BAK1 co-receptor dependent manner.

Key words

Arabidopsis, defense signaling, root development, DAMP, phytoytokines, secreted peptide, oxidative stress

Abbreviations

DAMP: Damage/Danger-Associated Molecular Pattern; PA: Phosphatidic Acid; PTMP: Post-Translationally Modified Peptide; CRP: Cysteine-Rich Peptide; ROS: Reactive Oxygen Species.

Introduction

In order to counter constant pathogen aggressions, plants have developed sophisticated perception and defense systems. These plant responses are regulated by complex networks involving regulatory proteins and hormones and are associated with massive changes in gene expression (Buscaill and Rivas, 2014). Among the involved actors, it has been shown that small secreted peptides play an important role through their direct interaction with pathogens or through their function in development and cell-to-cell communication involving ligand-receptor interactions (Murphy *et al.*, 2012; Marmioli and Maestri, 2014, Gust *et al.*, 2017). The secreted peptides derive from protein precursors having a shared N-terminal signal peptide which target the protein to the secretory pathway. They can be categorized into two major classes: (i) the small post-translationally modified peptides (PTMP) which are the targets of posttranslational maturations and are produced through a proteolytic processing and (ii) the cysteine-rich peptides (CRP) characterized by an even number of cysteine residues involved in intramolecular disulfide bonds (Tavormina *et al.*, 2015). Although they are mainly involved in plant growth and developmental processes, it has been shown that numerous genes encoding secreted peptides are also involved in plant defense mechanisms (Albert, 2013). For instance, the CRP class includes the antimicrobial peptides (such as knottins and defensins) which interact and disrupt the pathogen cell membrane (Goyal and Mattoo, 2014). Regarding PTMPs, families such as the phytosulfokines (PSK), CLE/CLV3, IDA/IDL or PSY are actors in processes regulating a large panel of plant-pathogen interactions (Rodiuc *et al.*, 2015; Lee *et al.*, 2011; Vie *et al.*, 2015; Shen and Diener, 2013). Among secreted peptides, those showing immunity-inducing activity have been classified as damage/danger associated molecular pattern, *i.e.* DAMPs (Heil *et al.*, 2012; Boller and Felix, 2009). Through the action of lytic enzymes, a pathogen can penetrate the plant cell wall; the cell wall fragments thus released into the apoplastic space can be perceived by neighboring cells, resulting in defense reactions. Oligalacturonides and cutin monomers are examples of non peptidic DAMPs which get released upon fungal infection (Fauth *et al.*, 1998). Their perception by neighboring cells elicits immunity response as well (De Lorenzo *et al.*, 2011). The small peptide *AtPep1* is a well-documented DAMP (Bartels and Boller, 2015). A first induction of *AtPep1* and other peptides of this family by wounding or pathogen attack has a positive feedback on the expression of its own precursors as well as defense marker genes that is thought to amplify defense signaling pathways (Huffaker and Ryan, 2007).

It is considered that only a small fraction of the gene space likely to encode signaling peptides has been described and their diversity appears to be largely underestimated (Matsubayashi, 2014). Indeed, the Arabidopsis genome contains more than a thousand genes harboring secreted peptide features for which the biological function is currently unknown (Lease and Walker, 2006 and 2010). This lack of data can be explained by the fact that this type of genes has only recently been detected due to their small size and their low sequence conservation (Silverstein *et al.*, 2007). Furthermore, the frequent functional redundancy inside these gene families (Matsubayashi, 2014) rendering mutant knock-out approaches less successful. The mining of previously published transcriptomes is an efficient way to explore this unknown gene-space and decipher functions of new genes for which, without reference, the inference of function by similarity cannot be applied. Based on transcriptome meta-analysis and bioinformatics predictions in a 'guilt by association' approach, we identified a peptide family, of which at least one member is involved in plant immunity and root development. This work describes the identification of a gene family specific to the Brassicaceae genus encoding putative secreted peptides. The functional characterization of *PROSCOOP12*, one of its members in Arabidopsis, shows that this small gene could act as moderator in the response to different pathogen aggressions and in root development presumably via controlling ROS detoxification. We illustrate that the small endogenous SCOOP12 peptide displays most properties of phytocytokines, processed and actively transported actors of endogenous danger signal without cellular damage (Gust *et al.*, 2017).

Materials and Methods

Plant material

Plant material used was wild-type *Arabidopsis thaliana* L. Heynh cultivar 6 Columbia (Col-0) as well as the cultivar Wassilewskija (Ws) and the mutants *proscop12* (T-DNA line FLAG_394H10 in Ws background, primers used for genotyping are detailed in **Table S1**) *bak1-4* (T-DNA line SALK_116202), *fls2* (Gomez-Gomez and Boller, 2000) and *pepr1/pepr2* described by Flury *et al.* (2013). The *proscop12* mutant in Col-0 background was created using the CRISPR-Cas9 approach. We searched *proscop12* gene-specific sgRNA and potential off-target sites in the Arabidopsis Col-0 genome using the Crispor Tefor program (<http://crispor.tefor.net>). The 20 bases long-sgRNA with the sequence AAGAACTTGACCCATTTTGG was used. Soil grown plants used for *Erwinia amylovora* and

Alternaria brassicicola inoculations as well as all *in vitro* plants (on Murashige and Skoog) were grown under short day conditions (photoperiod of 8h light at 22°C/16h dark at 21°C, with 70% of relative humidity). Plants used for all other assays were grown under long day conditions (photoperiod of 16h light at 22°C/8h dark at 21°C, with 60% relative humidity). *B. napus* (Darmor-bzh) and *L. esculentum* (Sweet Baby) were grown under short day conditions.

Plant inoculation with E. amylovora

Wassilewskija (Ws), Columbia (Col-0) and *proscop12* mutant in both genotypes were grown 5 weeks on soil. Four leaves of 20 plants were infiltrated with bacterial suspensions of wild-type strain of *E. amylovora* CFBP1430 at a concentration of 10^7 colony forming units (cfu/ml) in sterile water or with mock using a needleless syringe. Symptom severity was scaled as described in Degraeve *et al.* (2008). For symptom rating (for Ws and *proscop12*-Ws), at least 12 rosette leaves were used per condition in two biological replicates. Maximal symptoms appeared at 24 or 48hpi depending on biological replicates. Therefore, representative experiments are presented either at 24 or 48hpi. For bacterial counting (for Col-0 and *proscop12*-Col-0), samples were taken 3 days post infection using a cork-borer (d=5mm) to cut one leaf disc per infected leaf. Leaf discs were ground in sterile water, diluted and plated as droplets of 10µl on LB plates. Plates were incubated, and colonies were counted the next day. Bacteria of 32 leaves of WT and *proscop12* were extracted and quantified.

Seed contamination and leave infection by A. brassicicola

Fifty surface sterilized seeds per petri dish of Ws and *proscop12* were immersed in a solution containing *A. brassicicola* (strain abra43) with 10^3 conidia/ml, for one hour and dried under sterile conditions. Leaves of Ws wild-type and *proscop12* mutant were inoculated with 5µl of an *A. brassicicola* solution, with a concentration of 10^3 conidia/ml. Symptoms were observed six days after infection. Necrosis areas were quantified using ImageJ. The experiments were repeated three times.

Protection assay

Mature leaves of *A. thaliana* plants were infiltrated by needleless syringe infiltration with the indicated elicitor peptide or control solution and were kept under long day growth conditions for 24h. The *Pseudomonas syringae* pv *tomato* DC3000 strain was grown in overnight culture

on YEB medium plates supplemented with appropriate antibiotics. Cells were harvested from the plate and re-suspended in sterile 10mM MgCl and diluted to an OD600 of 0.02. Bacteria solution was needles syringe infiltrated into the pre-treated leaves. Plants were maintained at high humidity. Samples were taken using a cork-borer (d=8mm) to cut one leaf disc per infected leaf. Leaf discs were ground in 10mM MgCl, diluted to the indicated concentration and plated as droplets of 10 μ l on YEB plates with the appropriate selection. Plates were incubated at 28°C and colonies counted two hours after the infection (0 dpi) as well as 1 and 2 days post infection. Eight plants were infected for each pre-treatment and sampling time point. The experiment was performed two times with similar results.

Transcriptomic analysis

Microarray analysis was performed with the CATMA array v5 (Hilson *et al.*, 2004). Leaves were collected 24h after inoculation from two independent biological replicates. Total RNA was extracted using the Qiagen RNeasy kit according to the supplier's instructions. RNA integrity, cDNA synthesis, hybridization and array scanning were performed as described in Lurin *et al.* (2004). cDNA from leaves inoculated with *E. amylovora* were hybridized against cDNA of leaves inoculated with water collected at the same time-point. Statistical analysis was based on two dye swaps as described in Gagnot *et al.* (2008). To determine differentially expressed genes, a paired t-test on the log ratios was performed. Spots displaying extreme variance were excluded. The raw *p*-values were adjusted by the Bonferroni method, which controls the Family Wise Error Rate. We considered differentially expressed genes with a Bonferroni *p*-value ≤ 0.05 Gagnot *et al.* (2008).

Determination of gene expression by qPCR

Detached leaves of three weeks old plants were collected and floated for two hours in elicitor or control solution. After the treatment, material was frozen and ground in liquid nitrogen. RNA from 100 mg of tissue was extracted using the NucleoSpin RNA plant extraction kit (Macherey-Nagel Hoerd, France). The DNase treatment was performed according to the manufacturer's recommendations. Per PCR reaction, complementary DNA was synthesized from 10 ng of total RNA extract with oligo(dT) primers using Moloney Murine Leukemia Virus Reverse Transcriptase according to the manufacturer's instructions (Promega). For quantitative real-time reverse transcription PCR (qPCR) in a 96-well format, the Chromo4™

System (Bio Rad) was used. Expression was normalized to that of the gene *ACR12* (AT5G04740, because of its constant transcription profile upon elicitor treatments) using the qGene protocol (Muller *et al.*, 2002). All the gene-specific primers used are detailed in **Table S1**.

Seedling growth inhibition assay

Seedlings were germinated on MS agar and grown for 5 days before transferring one seedling per well to 24 well plates containing 500 μ l MS media or MS media supplied with the indicated elicitor peptide to a final concentration of 1 μ M (six replicates per elicitor peptide treatment). Photos were taken, fresh weight and root length were measures after 8 additional days. Root length of *proscop12* and WT plants was determined on vertical MS plates.

Elicitor peptides

Peptides of flg22 (QRLSTGSRINSAKDDAAGLQIA), *A. thaliana* Plant Elicitor Peptide 1 (AtPep1) (ATKVKAKQRGKEKVSSGRPGQHN), SCOOP12 (PVRSSQSSQAGGR), scSCOOP12 (GRPRSASSGSVQQ), SCOOP12 S5/7A (PVRSAQASQAGGR), SCOOP12 S5A (PVRSAQSQAGGR) and SCOOP12 S7A (PVRSSQASQAGGR) were obtained from Eurogentec SA (Angers, France) and diluted in water to the final concentration used for the assays.

Measurement of reactive oxygen species

For ROS assays leaf discs of three weeks old soil grown plants, were placed into each well of a white 96-well plate (Thermo Scientific, Waltham, USA) in 0,1 ml of water and kept in the dark overnight. For elicitation and ROS detection, horseradish peroxidase and luminol were added to a final concentration of 10 μ g ml⁻¹ and 100 μ M, respectively. Luminescence was measured directly after addition of elicitor peptides in a FLUOstar OPTIMA plate reader (BMG LABTECH, Offenburg, Germany).

Callose deposition

Leaf discs were vacuum infiltrated for 10 min with the indicated elicitor solution and kept floated in elicitor or control solution for 24h. After leaf discs were fixed and destained in 1:3 acetic acid/ethanol until leaf tissue was completely transparent. After washing the leaf discs in 150 mM K₂HPO₄ for 30 min, the plant material was stained for 2 h in 150 mM K₂HPO₄ and

0,01% aniline blue. Callose depositions were quantified with a Leica DM1000 microscope equipped with a Qimaging Micropublisher 3.3 RTV camera using a DAPI filter.

Cell culture conditions

A. thaliana cells were grown in a liquid MS based (Duchefa-Kalys, France) growth medium (pH 5.6) with the addition of 2,4-dichlorophenylacetic acid (0.2 mg/l), sucrose (30 g/l), and KH_2PO_4 (0,2 g/l). Cells were grown under continuous light ($200 \mu\text{E m}^{-2} \text{s}^{-1}$) on a rotary shaker and weekly sub-cultured to a fresh medium. For radiolabeling experiments 7-day-old cell suspensions were used.

Radioisotope labelling of phospholipids

Arabidopsis cells were aliquoted (7 mL) in individual flasks and kept for 3h under mild rotation for equilibration. Radioisotope labelling was done by the addition of $53 \text{ MBq.L}^{-1} [^{33}\text{P}]$ -orthophosphate. Lipids were extracted according Krinke *et al.* (2009). Lipids were separated by thin layer chromatography (TLC) using an acidic solvent system composed of chloroform:acetone:acetic acid:methanol:water (10:4:2:2:1, v/v) (Lepage, 1967) or in a solvent system composed of chloroform:methanol:ammonia:water (90:70:1:16, v/v) (Munnik *et al.*, 1994). Radiolabelled spots were quantified by autoradiography using a Storm phosphorimager (Amersham Biosciences, UK). Individual phospholipids were identified by co-migration with non-labelled standards visualized by primuline staining or by phosphate staining.

Accession numbers

Transcriptome data are available at Gene Expression Omnibus with the accession GSE22683. The samples used (including biological repetitions) are: GSM562282, GSM562283, GSM562284, GSM562285, GSM562286, GSM562287, GSM562288, GSM562289, GSM562294, GSM562295, GSM562296, GSM562297.

Results

Identification of the PROSCOOP gene family

Meta-analysis of CATMA micro-array data (Gagnot *et al.*, 2008) has previously highlighted several hundreds of non-annotated small protein-coding genes of unknown functions in

Arabidopsis (Aubourg *et al.*, 2007). Further we investigated the whole CATMA resource available at this time in order to identify new genes induced by various stresses for further functional analyses. Among them, *AT5G44585* caught our attention because of its highly informative profile: this gene was differentially expressed in 136 experiments (21%), being strongly induced in response to a large panel of biotic or oxidative stresses, *Erwinia amylovora* infection being in the top of them. Biological contexts were extracted from each CATdb experiment (<http://tools.ips2.u-psud.fr/CATdb>) and classified in 8 classes (**Fig. 1** and **Table S2**). It is noteworthy that no less than 70% of the complete transcriptomic response of *AT5G44585* could be summarized with three keywords: pathogen response, oxidative stress and root growth. Generally, we found this gene to be strongly up-regulated in most biotic and oxidative stress conditions, while it was down-regulated in conditions aiming at diminishing oxidative stress. Furthermore, *AT5G44585* exhibited a constitutive expression in roots in growth conditions but is down-regulated in numerous conditions affecting root elongation such as nitrogen starvation (Krapp *et al.*, 2011). This advocated for further exploration of this gene in oxidative stresses, root development, and in response to pathogen infections.

The screening of the Arabidopsis genome revealed that *AT5G44585* belongs to a small family of 14 unknown homologous genes with similar intron-exon structure (2 or 3 exons), encoding proteins ranging from 72 to 117 amino-acids (aa). Analysis of the N-terminal regions using the SIGNALP v4.1 (Nielsen, 2017) and the PREDOTAR v1.04 (Small *et al.*, 2004) software revealed a signal peptide targeting proteins to the endoplasmic reticulum to be present in all members of the family. DeepLoc v1.0 (Almagro *et al.*, 2017) predict an extracellular localization for the 14 proteins with scores ranging from 0.88 to 1. The 14 genes are organized in two tandemly arrayed clusters on chromosomes 1 and 5 (**Fig. 2A**). The largest 37 kb long gene cluster on chromosome 5 contains numerous vestiges of transposable elements (Helitron type) which could have impacted evolution of this family through local duplication events. Manual annotation revealed two additional yet non-annotated genes located between *AT5G44565* and *AT5G44568*. Both share significant similarities with the other tandemly arrayed homologs and cognate expressed sequence tags (ESTs) validate their transcription. Our manual annotation also led to the correction of the structure of *AT5G44570* in which an over-predicted 3' coding exon has been removed. The size of the proteins, the number and the organization of paralogs, the aa composition (notably the absence of cysteine) and the presence of a signal peptide are common features shared by the PTMP families previously

published (Matsubayashi, 2014). Furthermore, as described below, we identified a short conserved motif in the C-terminal region of these proteins, candidate to be mature functional peptides after proteolytic processing. For these reasons, this newly identified family has been named PROSCOOP as putative precursors of SCOOP peptide (Serine riCh endOgenOs Peptide). The genes are termed *PROSCOOP1* to *14* (*AT5G44585* being *PROSCOOP12*) and the corresponding mature peptides named SCOOP1 to 14 (**Fig. 2A**).

Previously reported RNA-seq approaches (Hruz *et al.*, 2008) allowed us to broaden our transcriptome analysis to the *PROSCOOP* family members that were missing on the micro-arrays (only 4 of them are present in the Affymetrix Ath1 chip). We could confirm the regulation of their transcription in several stress conditions and organs (**Fig. 2B**). These data show a large diversity of transcription profiles inside this family suggesting its involvement in different biological functions. Notably, *PROSCOOP12* shows a distinct transcription profile as it is among the minority of paralogs to be highly induced by different pathogen aggressions and expressed in the whole root system.

In order to assess the evolutionary conservation of the PROSCOOP family, an extensive BLASTP search for homologs in GenBank was carried out. We identified this family in several Brassicaceae genomes reaching from *Eutrema salsugineum* to *Camelina sativa* and the number of identified homologs in these genomes ranged from 1 to 13. Outside Brassicaceae genus, no similar proteins could be detected despite low stringency searches. The phylogenetic tree built from the multiple alignment of the 74 identified *PROSCOOP* homologs shows that gene duplications occurred before speciation of the 8 different Brassicaceae species (**Fig. S1**).

In order to identify divergent yet still conserved smaller regions, the MEME algorithm (Bailey *et al.*, 2015) was used, excluding full length alignments, on the 74 identified homologs. This sensitive approach allowed the identification of two significantly conserved 11 aa-long motifs (**Fig. 3**). These motifs are good candidates for functional mature peptides (or a part of them) following the putative proteolytic processing of the corresponding precursor. Indeed, both motifs are proline-, serine-, arginine- and glycine- rich, as in previously described PTMP families such as CLV3/CLE (Betsuyaku *et al.*, 2011), IDA (Vie *et al.*, 2015), PIP (Hou *et al.*, 2014) and CEP (Roberts *et al.*, 2013). The motif 1 is more ubiquitous than the motif 2 since it was detected in 72 sites (e-value of $9.8e-213$) compared to 39 sites (e-value of $3.4e-179$) out of

the 74 PROSCOOP homologs. Therefore, we have focused our downstream functional analysis on motif 1 (**Fig. 3**), named SCOOP thereafter.

PROSCOOP12 is co-expressed with genes involved in hormone signaling and defense

In order to make a first assessment of the potential biological relevance of *PROSCOOP12* and to predict its putative functional partners, we further mined previously published Arabidopsis transcriptome data (Gagnot *et al.*, 2008). Based on the assumption that genes with related biological functions are likely to be co-expressed (Schöner *et al.*, 2007), we used the results of the Gaussian mixture model-based clustering method from the GEM2Net resource (Maugis *et al.*, 2009; Zaag *et al.*, 2015). The *PROSCOOP12* gene was found to be co-expressed with 83 genes in a set of experimental samples gathering biotic stress triggered by necrotrophic bacteria and fungi. This cluster of 83 genes has been enriched by the integration of functional partners based on co-citations, protein-protein interactions and common biological pathways) in using TAIR, the Arabidopsis interactome (Arabidopsis Interactome Mapping Consortium, 2011) and the STRING database (Szklarczyk *et al.*, 2017). This step resulted in a network of 117 genes (**Tables S3A and S3B**) mainly focused on hormone crosstalk (especially SA/JA signaling), pattern-triggered immunity (PTI), brassinosteroid and phenylpropanoid pathways and nitrogen metabolism (**Fig. S2**). Out of 117 genes, 53 are involved in response to stimulus (GO:0050896, *fdr* 1.31e-11), among them, 26 genes are classified in defense response (GO:0006952, *fdr* 5.72e-10), and 14 in transmembrane signaling receptor activity (GO:0004888, *fdr* 1.41e-09). Numerous key defense actors were found to be clustered with *PROSCOOP12* such as the *NIMIN1*, *IOS1*, *NHL6*, *MLO12*, *FRK1*, *LECRKA4.1*, *CRK13*, *HA2* genes and the *WRKY-11*, *-14*, *-18*, *-22*, *-60* and *-70* transcription factors. This relational network contains two other genes encoding PTMPs, namely *PROVIR10* and *PSK4*, and two PTMP receptor kinases, *PSKR1* and *PSY1R* that are involved in root development and modulation of SA/JA defense responses (Mosher *et al.*, 2013). *PROVIR10* has been found to correlate positively with disease triggered by necrotrophic pathogens (Dobón *et al.*, 2015) and *PSK4* encodes a phytosulfokine, one of the peptide growth factors involved in disease establishment (Rodiuc *et al.*, 2015). This approach led us to explore the role of *PROSCOOP12* and its SCOOP12 peptide regarding fungal and bacterial infections.

PROSCOOP12 is involved in pathogen defense and root development

Screening Arabidopsis mutant collections (Dèrozier *et al.*, 2011) we identified a T-DNA mutant *proscop12* in the Wassilewskija (Ws) background. Homozygous mutant plants did not transcribe *PROSCOOP12* (**Fig. S3**). Based on *PROSCOOP12* transcription induction in the presence of different pathogens, (**Fig. 1, Fig. 2B** and **Table S2**), the analysis of co-expressed putative partners, and its putative role as a secreted DAMP, we decided to challenge the mutant with the necrogenic bacterium *Erwinia amylovora* and the necrotrophic fungus *Alternaria brassicicola*.

Compared to wild-type plants, *proscop12* displayed a higher tolerance to *E. amylovora* induced cell death as observed by a reduction of necrotic symptoms in leaves (**Fig. 4A**). This phenotype has only been observed in *wrky70* (Moreau *et al.*, 2012). Like *WRKY70*, *PROSCOOP12* acts as a negative regulator of defense against this bacterium. The transcription factor *WRKY70* is known to positively regulate *WRKY60* and it is involved in the JA/SA crosstalk (Li *et al.*, 2004). Notably, these two genes have been found clustered with *PROSCOOP12* in our gene network analysis (**Fig. S2**). Then, we performed a micro-array transcriptomic comparison of *proscop12* versus wild-type following bacterial inoculation. Results show that 3,731 genes were differentially expressed in wild-type in response to *E. amylovora*, and 4,125 in *proscop12*. Despite the difference in symptom intensity, the vast majority of the bacteria-responsive genes did not display significant differences in both lines. Indeed, only 131 genes displayed a significantly different expression (Bonferroni *p*-value 5%) between wild-type and *proscop12* infected plants (**Table S4**): 126 up-regulated and 5 down-regulated genes, these latter corresponding only to hypothetical proteins or pseudogenes.

The 126 up-regulated genes that may contribute to the difference in symptoms between *proscop12* and wild-type were challenged by functional annotation adding literature references to GO terms to provide additional information (**Table S4**, summarized in **Fig. 4B**). Indeed, 45% of them are connected to defense response (such as *HR4*, *SQP1*, *AED1*, *MKK2*, *HD2B*, *NPR3*) and/or protection against oxidative stress (such as *ALDH24B*, *BiP2*, *APX1*, *ATOM1*, *APR1*, *PER50*). Moreover, 18% were related to response to other stresses, mainly oxidative stress, and 10% could have indirect links with stress since involved in processes such as cell wall modifications or proteolysis. Only 13% could not be related to the phenotype, often because their function is currently unclear. Finally, the remaining 14% are unknown genes. The high percentage of genes directly related to protection to oxidative stress supports the hypothesis of a relationship between *PROSCOOP12* and the control of ROS production.

The response of *proscop12* to a necrotrophic fungus infection was assessed using the Arabidopsis – *A. brassicicola* pathosystem (Pochon *et al.*, 2012). *A. brassicicola* inoculation of rosette leaves produced similar symptoms in wild-type and *proscop12* genotypes (**Fig. S4**). Because seedling infection by *A. brassicicola* is mainly caused by seed transmission, we have also observed the fungus colonization during germination of infected seed lots under controlled conditions. Two days after sowing, *proscop12* showed a significantly lower rate of germinating seeds prone to *A. brassicicola* infection compared to the wild-type (**Fig. 4C**). Because our transcriptome analysis suggested that *PROSCOOP12* may play a role in root development (**Fig. 1** and **Fig. 2B**), we compared the root lengths of wild-type and *proscop12* plants. Indeed, *proscop12* plants developed significantly longer roots than control plants (**Fig. 5A** and **Fig. 5B**). No significant difference was observed between wild-type and *proscop12* regarding the seedling fresh weight (**Fig. 5C**).

A second *proscop12* line was obtained in the Col-0 background using a CRISPR-Cas9 approach. The frameshift obtained in the first exon disrupts the coding frame 10 aa after the editing event, upstream the conserved motif. The phenotypes previously observed with the Ws *proscop12* mutant were confirmed in this Col-0 mutant line (**Fig. S5**).

The SCOOP12 peptide has main features of DAMPs

The structural features of the *PROSCOOP12* protein suggested that it should be classified as a secreted PTMP. Besides, at the functional level, its transcriptional behavior suggested that it may play a role as a DAMP. Indeed, the induction of *PROSCOOP12* expression by a large panel of biotic stresses and the root phenotypes identified in the *proscop12* mutant revealed some analogies with the *AtPROPEP1* and *AtPROPEP2* genes which are the precursors of the *AtPep1* and *AtPep2* peptides respectively, well-characterized DAMPs (Bartels and Boller, 2015). Likewise, both genes are also induced by biotic stress (Huffaker *et al.*, 2006) and the *AtPep1* DAMP is involved in root development since the overexpression of *AtPROPEP1* and *AtPROPEP2* causes significantly longer roots (Huffaker *et al.*, 2006). Therefore, we wanted to test if *PROSCOOP12* encodes for peptide that may act as a DAMP by comparing it to *AtPep1*.

The SCOOP12 peptide induces immune responses in Arabidopsis

Based on the identification of the conserved motif 1 (**Fig. 3**), a putative mature peptide *SCOOP12* was defined (PVRSSQSSQAGGR) from *PROSCOOP12* and synthesized in order to

explore its biological function. Despite the non-predictable post-translational modifications, we tested the exogenous application of the synthetic SCOOP12 peptide as previously described for CLE and RGF PTMP families (Murphy *et al.*, 2012; Matsuzaki *et al.*, 2010; Whitford *et al.*, 2012). Treatment of plants with SCOOP12 induced a wide range of long- and short-term immune responses (**Fig. 6**). One of the fastest defense responses is the production of ROS (Torres *et al.*, 2006). We show here that SCOOP12 induced a more intensive ROS burst compared to *AtPep1* but weaker than *flg22* (**Fig. 6A**). Next, we wanted to study the effect of SCOOP12 on genes closely linked to early defense mechanisms. *FRK1* has previously been shown to be induced by pathogens, elicitors, salicylic acid (Asai *et al.*, 2002; Boudsocq *et al.*, 2010) and *AtPep1* (Flury *et al.*, 2013). Furthermore, our co-expression network approach identified a co-expression of *PROSCOOP12* with *FRK1* (**Fig. S2**). Therefore, we measured the *FRK1* expression level in detached leaves floating for 2h in solutions supplemented by SCOOP12 or *AtPep1*. Compared to controls, *AtPep1* and SCOOP12 treatments resulted in a 15-fold and 8.5-fold increase in *FRK1* expression, respectively (**Fig. 6B**). The deposition of callose is also known to be triggered by DAMPs (Luna *et al.*, 2011). Callose staining after 24h of treatment with the elicitor peptides showed that SCOOP12 induces a callose deposition, yet at a weaker level compared to *flg22* or *AtPep1* (**Fig. 6C** and **Fig. 6D**). One of the long-lasting defense responses is an inhibition of growth caused by the elicitor (Krol *et al.*, 2010). Our results indicate that perception of SCOOP12 also leads to an arrest of growth. The effect is comparable to the *flg22* and the *AtPep1* DAMP (**Fig. 6E**, **Fig. 6F** and **Fig. 6G**).

In order to demonstrate the specificity of SCOOP12 sequence, we synthesized a peptide based on a randomized version of the same amino acids and tested plant responses to this scrambled SCOOP12 (*scSCOOP12*). Furthermore, we synthesized peptides with double alanine replacements (SCOOP12 S5/7A) and single replacements (SCOOP12 S5A; SCOOP12 S7A) to test the importance of the two highly conserved serine residues on positions 5 and 7 of SCOOP12 (**Fig. 3**) for its activity. Plants treated with *scSCOOP12* as well as with the modified peptides did not show seedling growth inhibition. Total seedling fresh weight as well as root length were not different from that of control plants (**Fig. 7A**). Finally, treatments with *scSCOOP12*, SCOOP12 S5/7A and SCOOP12 S5A did not induce a ROS burst and only SCOOP12 S7A resulted in a low, still significant ROS burst (**Fig. 7B**). These results highlight the importance of the amino acid order and the highly conserved serine residues for the perception of SCOOP12 by the plant.

Next, we wanted to test the conservation of plant responses to SCOOP12. For that purpose, plants were selected in which we identified *PROSCOOP* homologues (*Brassica napus*, **Fig. S1**) and plants that do not contain this gene family (*Nicotiana benthamiana* and *Lycopersicon esculentum*). We measured ROS production following application of SCOOP12 in these plants and included flg22 as a positive control. We detected a ROS burst caused by flg22 in all four plant species. On the other hand, SCOOP12 only resulted in a ROS burst in *A. thaliana* and at a lower, yet still significant level, in *B. napus* (**Fig. S6**). SCOOP12 seems to be similar enough to its closest *B. napus* homolog (BNCDY22858 with the motif FAGPSSSGHGGGR) to trigger a ROS burst. Therefore, only the two plant species containing homologues of the *PROSCOOP* gene-family members, showed a response to SCOOP12 treatments.

Pre-treatment with the SCOOP12 peptide protects Arabidopsis against Pseudomonas infection
It has previously been shown that priming of plants with the flg22 elicitor as well as with oligogalacturonides could result in enhanced tolerance against subsequent bacterial infections. For instance, plants pre-treated with these elicitors showed significantly reduced lesion size following an infection with *Botrytis cinerea* (Raacke *et al.*, 2006; Ferrari *et al.*, 2007). Using a similar assay, we found that plants pre-treated with flg22 as well as with SCOOP12 and AtPep1 were less susceptible to *Pseudomonas syringae* pv. *tomato* DC3000 infection (**Fig. 8**). The effect of the two endogenous peptides SCOOP12 and AtPep1 was weaker than flg22, which is consistent with the fact that flg22 induced stronger defense response compared to SCOOP12 (**Fig. 6A** and **Fig. 6C**).

SCOOP12 and AtPep1 induce the expression of several PROSCOOP genes

It has previously been shown that small endogenous peptides can induce the expression of their own precursors resulting in a positive feed-back loop. For instance, expression of several *PROPEP* genes can be induced by different AtPep peptides (Huffaker and Ryan, 2007). This led us to investigate the change in steady state transcript level of all 14 *PROSCOOP* family members after SCOOP12 exposure. Moreover, we decided to add AtPep1 in our assay for comparison since it is also known to induce the transcription of another peptide precursor, *prePIP1* (Hou *et al.*, 2014). The results show that *PROSCOOP* 2, 7, 8, 12 and 13 are upregulated by the AtPep1 treatment (**Fig. S7**). Most importantly, the direct precursor *PROSCOOP12* is upregulated by SCOOP12 in comparison to the control treatment (**Fig. S7L**). Therefore, there

is a positive feedback loop linking SCOOP12 to its precursor *PROSCOOP12* but also of other members of the *PROSCOOP* family such as *PROSCOOP7*. However, SCOOP12 did not induce the expression of *PROPEP1* (**Fig. S70**). These results suggest that there is a feedback loop of SCOOP12 to its precursor and to *PROSCOOP7* and that AtPep1 is capable of inducing five members of the *PROSCOOP* family.

The BAK1 co-receptor is involved in SCOOP12 perception

A well characterized co-receptor of several receptors of small peptides is BRI1-associated kinase1 (BAK1). Interaction of BAK1 with receptor-like kinases that act as elicitor receptors, was proposed to be due to conformational changes occurring after ligand binding which results in the formation of the receptor complex (Chinchilla *et al.*, 2009; Liu *et al.*, 2017). To test if BAK1 is involved in the perception of SCOOP12, a seedling growth inhibition assay was performed on *bak1-4* plants. Compared to wild-type controls, *bak1-4* plants did not display any significant growth inhibition upon SCOOP12 treatment (**Fig. 9A**). The same approach was carried out on *fls2* (the flg22 receptor) and *pepr1/pepr2* plants. Contrary to BAK1, our results suggest that these receptors are not involved in the perception of SCOOP12 (**Fig. 9B** and **Fig. 9C**).

SCOOP12 rapidly activates phospholipid signaling pathways in Arabidopsis cell suspensions

Lipid signaling pathways act as multifunctional regulatory mechanisms in plants. They incorporate several groups of inducible enzymes that convert membrane phospholipids into signaling molecules. Phosphatidic acid (PA) is a well-known biologically active lipid that is produced in response to numerous hormonal and stress signals including, notably, flg22 (van der Luit *et al.*, 2000). We demonstrate that application of SCOOP12 induces an accumulation of PA in Arabidopsis cell suspensions (**Fig. 10A**). This effect is observed as early as 5 min following SCOOP12 application in a low concentration of 100nM (**Fig. 10B** and **Fig. 10C**). The scSCOOP12 had no effect on PA accumulation. Two modes of PA accumulation are known: Phospholipase D (PLD)-dependent via direct hydrolysis of membrane phospholipids and diacylglycerol kinase (DGK)-dependent via phosphorylation of diacylglycerol (DAG). In our experiment a labelling protocol that favors visualization of DGK-derived PA was used (Arisz and Munnik, 2013). Phosphatidylinositol 4,5-bisphosphate (PIP₂) is a substrate to phosphatidylinositol-specific phospholipase C (PI-PLC) that produce DAG. We have also observed that the level of PIP₂ is transiently reduced following SCOOP12 treatment (**Fig. 10B**). These results suggest that SCOOP12 initiates a signaling cascade implicating PI-

PLC (causing the depletion of PIP₂) and subsequent production of PA via phosphorylation of DAG by DGK.

Discussion

Considered jointly, our transcriptome, mutant phenotyping and peptide assay results allow us to propose a model explaining the roles of the SCOOP12 peptide in Arabidopsis (**Fig. 11**). The induction of numerous genes involved in the protection against oxidative stress such as peroxidases, glutathione transferase, phenylpropanoid synthases in *proscop12* in response to *E. amylovora* infection (**Table S4**) might indicate that its lack of expression could result in a decrease in H₂O₂ levels. This could impair *E. amylovora* progression in leaves, which is known to induce H₂O₂ production in plants in order to promote cell death and invade plant tissues (Venisse *et al.*, 2001; Degraeve *et al.*, 2008). In parallel it is known that antioxidant responses in roots decrease the H₂O₂ level in the elongation zone, thereby contributing to root growth (Dunant *et al.*, 2007; Tsukagoshi *et al.*, 2010). The constitutive expression of *PROSCOOP12* in roots (**Fig. 2**) could therefore contribute to higher levels of H₂O₂ and act as a moderator of root elongation under normal conditions. This is consistent with the greater root length observed in *proscop12* (**Fig. 5**) and with the decrease of *PROSCOOP12* expression in roots in conditions leading to root lengthening such as nitrogen starvation (**Table S2**).

In addition to its function in root elongation, we found *PROSCOOP12* to be involved in response to biotic stress in aerial parts where its transcription is strongly induced in presence of pathogens (**Fig. 1** and **Fig. 2B**). This induction triggers a ROS burst, putatively through the inhibition of the antioxidant responses and then participates to the increase of H₂O₂ level in the infected tissues. This mechanism occurs when we apply the synthetic SCOOP12 peptide on seedlings, as illustrated by its induction of ROS burst, transcription of the *FRK1* defense gene, and callose deposition in leaf cells (**Fig. 6**). SCOOP12-induced PA production (**Fig. 10**) can be a part of a signaling cascade implicating several PA-binding proteins (Pokotylo *et al.*, 2018). PA binds NADPH oxidase isoforms D and F and stimulates NADPH oxidase activity in guard cell protoplasts (Zhang *et al.*, 2009). That is why PA production is likely to be upstream of ROS accumulation observed in response to SCOOP12. We have shown that the effects of SCOOP12 are BAK1-dependent (**Fig. 9A**). It is known that the activity of BAK1 in receptor complexes is dependent on its phosphorylation state and is controlled by protein phosphatase 2A (PP2A) (Segonzac *et al.*, 2014). PA interacts with the scaffolding A1 subunit

of PP2A, tether it to membranes and induce its activity (Gao *et al.*, 2013). This process was highlighted in connection to PIN1 dephosphorylation by PP2A in auxin signaling cascade. However similar reactions are to be expected for BAK1 dephosphorylation in PAMP/DAMP receptor complexes and indicate that they may act as intrinsic part of SCOOP12 regulatory cascade in plants.

The negative action of SCOOP12 on antioxidant response is consistent with the reduction of symptoms observed in the *proscoop12* defective mutant in presence of the necrogenic bacterium *E. amylovora* (Degrave *et al.*, 2008). In this case, the suppression of *PROSCOOP12* seems to enhance the protection against oxidative stress, thus hampering bacterial development in infected Arabidopsis leaves.

The comparison of the PROSCOOP family with other previously published genes encoding such secreted peptides highlights numerous shared features but also interesting specificities. At the structural level, the PROSCOOP proteins distinguish themselves by the absence of a highly conserved C-terminal region. Indeed, the motifs detected with the MEME tool are quite divergent compared to the other PTMP precursors (Matsubayashi, 2011). This divergence may explain the fact that no PROSCOOP homologs could be detected outside the *Brassicaceae* genomes. This restricted phylogenetic profile is opposite to the other described secreted peptides which are conserved both in monocots and eudicots. Furthermore, contrary to the majority of the known PTMPs, the conserved motifs are not localized at the C-terminal extremity of their precursors, and their maturation could involve two steps of proteolytic processing or a trimming step (Matsubayashi, 2011). Out of the 14 Arabidopsis PROSCOOP proteins, three include two duplicated SCOOP motifs (**Fig. 3**), reminiscent of the previously described cases of the CEP and PIP families (Roberts *et al.*, 2013; Vie *et al.*, 2015) and also of the CLE18 protein in which each copy of the conserved CLE motifs has a specific function (Murphy *et al.*, 2012). The motif composition classifies SCOOP in the superfamily of 'SGP-rich peptide' among PIP, CLE, IDA, PEP and CEP families (Hou *et al.*, 2014). At the functional level, the triggering of ROS burst, *FRK1* transcription and callose deposition move SCOOP12 close to the cytosolic AtPEP and apoplastic PIP families (Huffaker *et al.*, 2006). Our results suggest a functional link between AtPep1 and SCOOP12 since both peptides induce the transcription of *PROSCOOP12* (**Fig. S7L**). This collaboration between different peptide families has also been described with AtPEP1 and PIP1 which act cooperatively to amplify triggered immunity. Furthermore, the signaling induced by AtPep1 (Schulze *et al.*, 2010), PIP1 (Hou *et al.*, 2014)

and SCOOP12 (**Fig. 9A**) is dependent on the BAK1 co-receptor. In addition to their role as amplifiers of the immune response, these peptides are involved in root development but via different mechanisms. The overexpression of the PIP1 precursor or its exogenous application inhibits Arabidopsis root growth as described for CEP (Roberts *et al.*, 2013) and SCOOP12 peptide (**Fig. 6F**). On the other hand, the constitutive overexpression of *PROPEP1* increases the root development (Huffaker *et al.*, 2006) whereas *AtPep1* treatment inhibits root growth (Poncini *et al.*, 2017). Acting as growth factors and contrary to SCOOP12, the PTMP PSK and PSY1 are involved in root elongation (Amano *et al.*, 2007; Matsuzaki *et al.*, 2010). These comparisons show that despite common structural and functional characteristics, the SCOOP family is different from previously described secreted peptides. The divergence observed in the C-terminal sequence of PROSCOOP proteins suggest a large broad of biological functions through a diversity of receptors which will be the targets of future studies.

In conclusion, SCOOP12 belongs to a new family of putatively secreted peptides specific to the Brassicaceae species. At the functional level, such secreted peptides are classified as phytocytokines (such RALFs, systemin and PIPs), *i.e.* secondary endogenous danger signal. Indeed, this classification (Gust *et al.*, 2017) distinguishes them from classical DAMPs (primary endogenous danger signals) which are passively released from injured tissue without biosynthesis and secretion processes. Through its negative action on antioxidant responses and its positive effect on PA/ROS production (PLC pathway), SCOOP12 could play a role in the moderation of defense responses, as well as root elongation, to prevent unnecessary energy loss in a 'trade-off' fashion (Walters and Heil, 2007). The functions of such plant secreted peptides at the boundaries of development- and stress-signaling pathways open the way to future strategies that jointly consider product quality/quantity and new resistance traits.

Supplementary data

Supplementary Figure S1. Phylogenetic tree of *PROSCOOP* homologs. The tree was built with the neighbor-joining method from the multiple alignment of 74 homologous Brassicaceae proteins.

Supplementary Figure S2. Relational annotation of genes co-expressed with *PROSCOOP12* and their functional partners.

Supplementary Figure S3. Confirmation of absence of transcription in the *proscoop12* T-DNA knock-out line by RT-PCR.

Supplementary Figure S4. Effect of *A. brassicicola* infection on *proscop12* leaves.

Supplementary Figure S5. Confirmation of *proscop12* mutant phenotype in a second genotype.

Supplementary Figure S6. ROS burst measurements on selected plant species treated with SCOOP12.

Supplementary Figure S7. Transcriptional response of the *PROSCOOP* gene family to SCOOP12 and *AtPep1*.

Supplementary Table S1. Gene-specific primer sequences used for mutant genotyping and qPCR analysis of all the *PROSCOOP* genes.

Supplementary Table S2. List of the 136 comparisons in which transcription of *AT5G44585* was deregulated in CATdb (<http://tools.ips2.u-psud.fr/CATdb>). The *AT5G44585* gene is tagged by the CATMA5A40400 probe. Column legends: CATdb project references (providing Gene Expression Omnibus accessions and complete description of samples), project titles, “yellow” and “blue” sample names, organs, Y/B ratios, phenotype keyword extracted from the project summary in CATdb pointing the expected phenotype, phenotype classes and color codes used in **Fig. 1** (1.1: pathogen infections, 1.2: oxidative stress, 1.3: abiotic stresses, 1.4: JA-SA related mutants, 2: root growth, 3: hypocotyl growth, 4: silencing mutants, 5: various experiments).

Supplementary Tables S3. List of 117 genes involved in the relational annotation of *PROSCOOP12* (in addition to Fig. S2). Their protein name and complete functional annotation (from TAIR10 and/or literature) are indicated (Table S3A). Putative partner genes have been found co-expressed with *PROSCOOP12* (according the GEM2Net resource) or have been added in the network by curated co-citations in literature, pathways, and protein-protein interactions (STRING, Arabidopsis Interactome, KEGG...). The type and the source of the each network edge are detailed in Table S3B.

Supplementary Table S4. Transcriptomic comparison of *proscop12* and wild-type plants during *E. amylovora* infection. Column legend: CATMA probe ID, Arabidopsis gene ID, gene name according TAIR10, annotation according gene ontology (molecular function, biological process, cellular component), results of microarray hybridizations with raw intensities, Log2 ratio and Bonferroni *P*-value (Wt: wild-type, Sc12: *proscop12*, Ea: *E. amylovora* infection, H2O: control without *E. amylovora*), functional categories with the color code used in the

Figure 4B, keywords regarding functional pathways and references for justification. Pseudo color scale representing the differential expression is described in the first line of the table.

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Figure 1

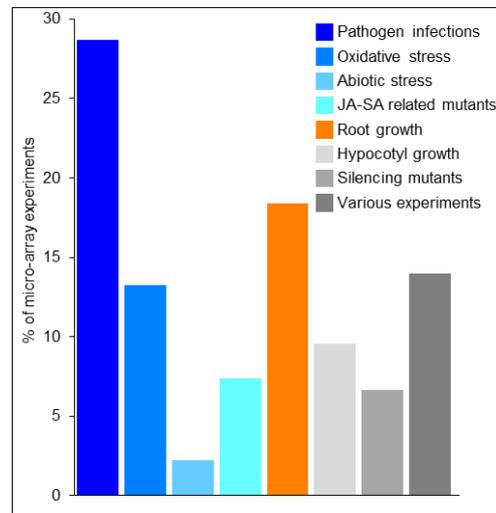


Fig. 1. Synthesis of the results from the 136 experiments in which *AT5G44585* was significantly deregulated (Bonferroni p-value <5%) within the CATdb resource, then sorted in 8 classes: pathogen infections, oxidative stress, abiotic stresses, JA-SA related mutants, root growth, hypocotyl growth, silencing mutants, various experiments. The whole set of results is detailed in **Table S2**.

Figure 2

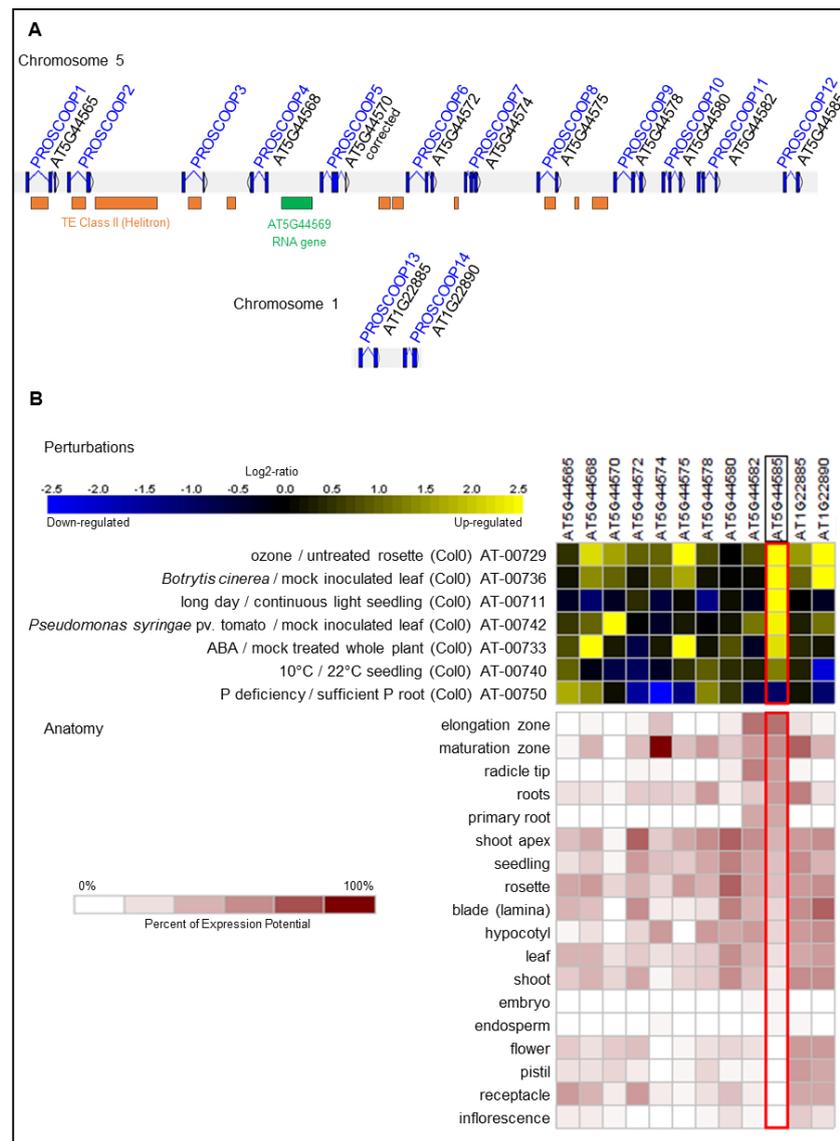


Fig. 2. The *PROSCOOP* family. (A) Gene organization: coding exons and introns are represented by blue boxes and blue broken lines respectively. Remains of transposable elements (Helitron type) are represented by orange boxes and the green one indicates a putative non-coding RNA of unknown function. The TAIR gene names and corresponding *PROSCOOP* nomenclature are indicated. *PROSCOOP2* and *PROSCOOP3* are not annotated in the last TAIR version but are confirmed by the ESTs EG446167, EG448031, EG446890 and CB253842. (B) Transcription of *PROSCOOP* family: significant (p -value<0.05) differential expression induced by specific perturbations (upper panel) and transcription level in different *Arabidopsis* organs (lower panel) are based on RNA-seq data obtained from the Genevestigator platform (Hruz *et al.*, 2008). The *PROSCOOP12* gene is indicated by a red frame.

Figure 3

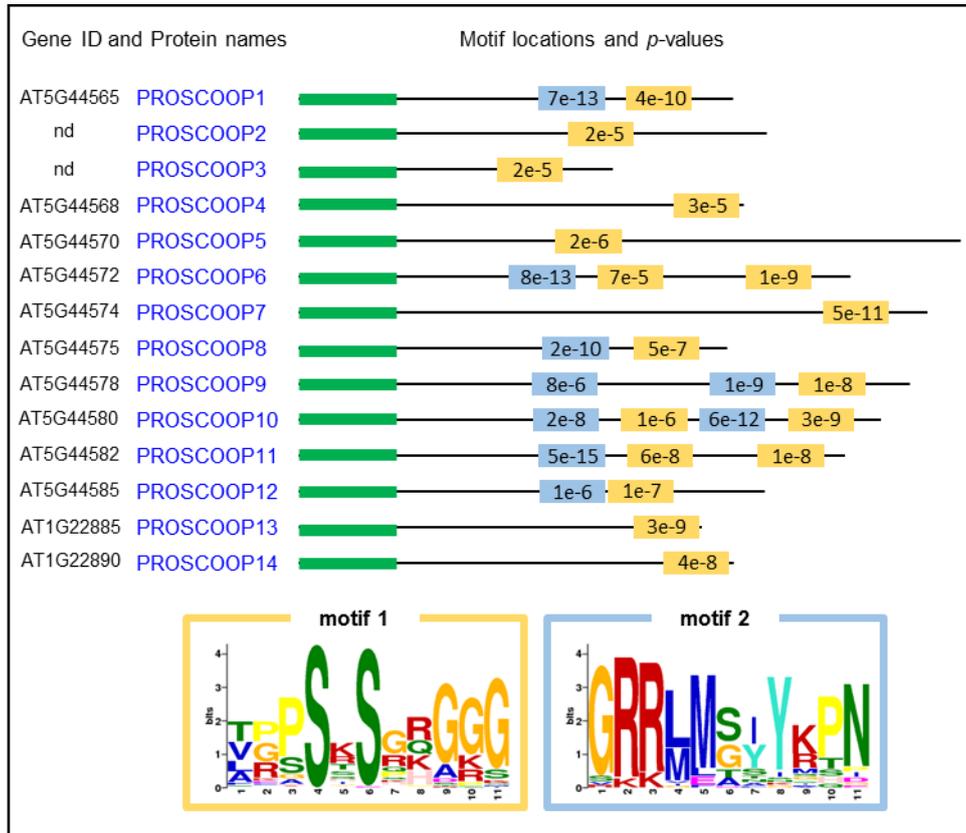


Fig. 3. Conserved motifs identified in the PROSCOOP family proteins. The MEME v4.8.1 algorithm (parameters -nmotifs 3 -minw 6 -maxw 12) was run on the 74 homologous PROSCOOP proteins found in Brassicaceae genomes. P-values and motif locations are only shown for the 14 members from Arabidopsis. A third motif corresponding to the cleavage site of the signal peptides (green boxes) has also been highlighted by MEME and fits with SIGNALP v4.1 predictions.

Figure 4

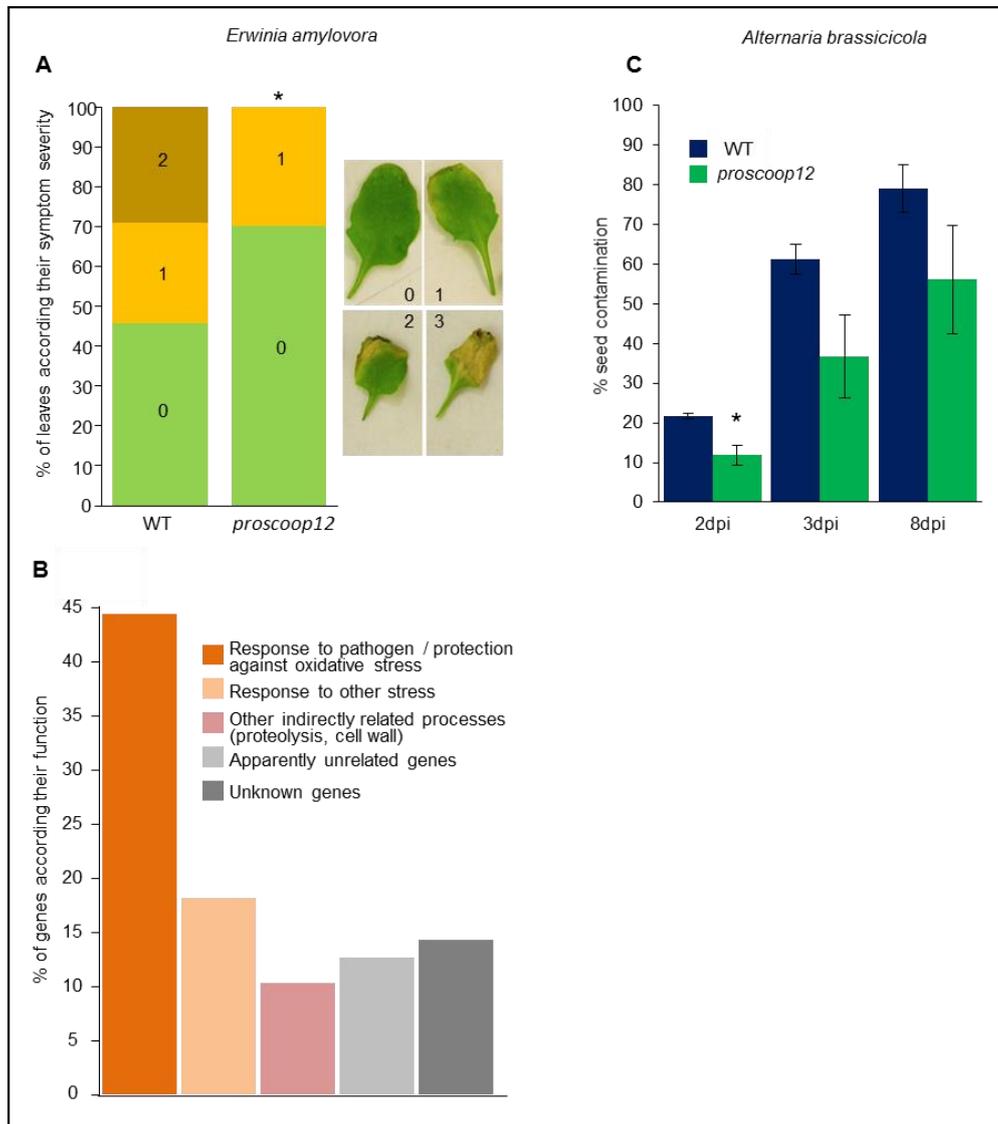


Fig. 4. Mutant phenotype in response to *E. amylovora* and *A. brassicicola* infections. (A) Effect of *E. amylovora* infection on *proscoop12* mutant. Symptom scale used (0 to 3) is illustrated on the right. The asterisk indicates a significant difference from symptom severity in wild-type leaves inoculated with *E. amylovora* (Mann and Withney, $\alpha = 0.05$). (B) Distribution of the 126 genes upregulated in *proscoop12* versus wild-type inoculated by *E. amylovora* according their functional annotation. The complete results of this transcriptome approach are in **Table S4**. (C) Effect of *A. brassicicola* seed infection on *proscoop12* during germination 2, 3 and 8 days post-imbibition. Significant differences, according to Student's t-test results: *, $P < 0.05$.

Figure 5

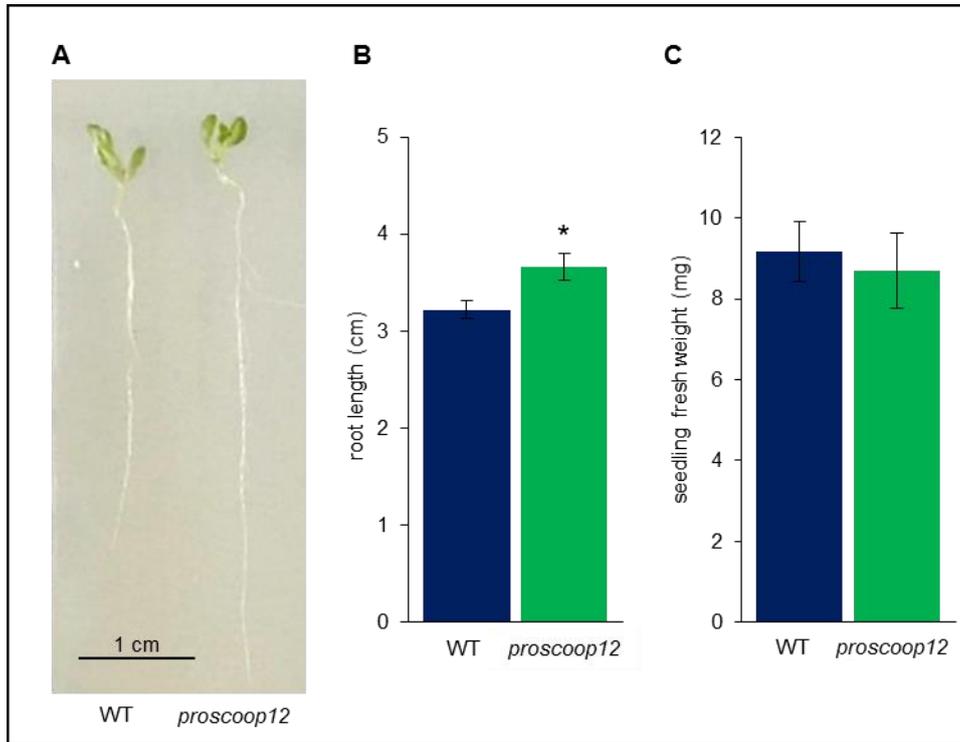


Fig. 5. Phenotypic comparison between *proscoop12* and wild-type plants. (A, B) Root growth phenotypes determined after 10 days. Student's t-test revealed that the different root length between wild-type and mutant is highly-significant (*, $P < 0.05$). (C) Seedling fresh weight determined after 10 days. Bars show the combination of 2 biological repetitions (25 seedling each) and error bars show \pm SE of the mean.

Figure 6

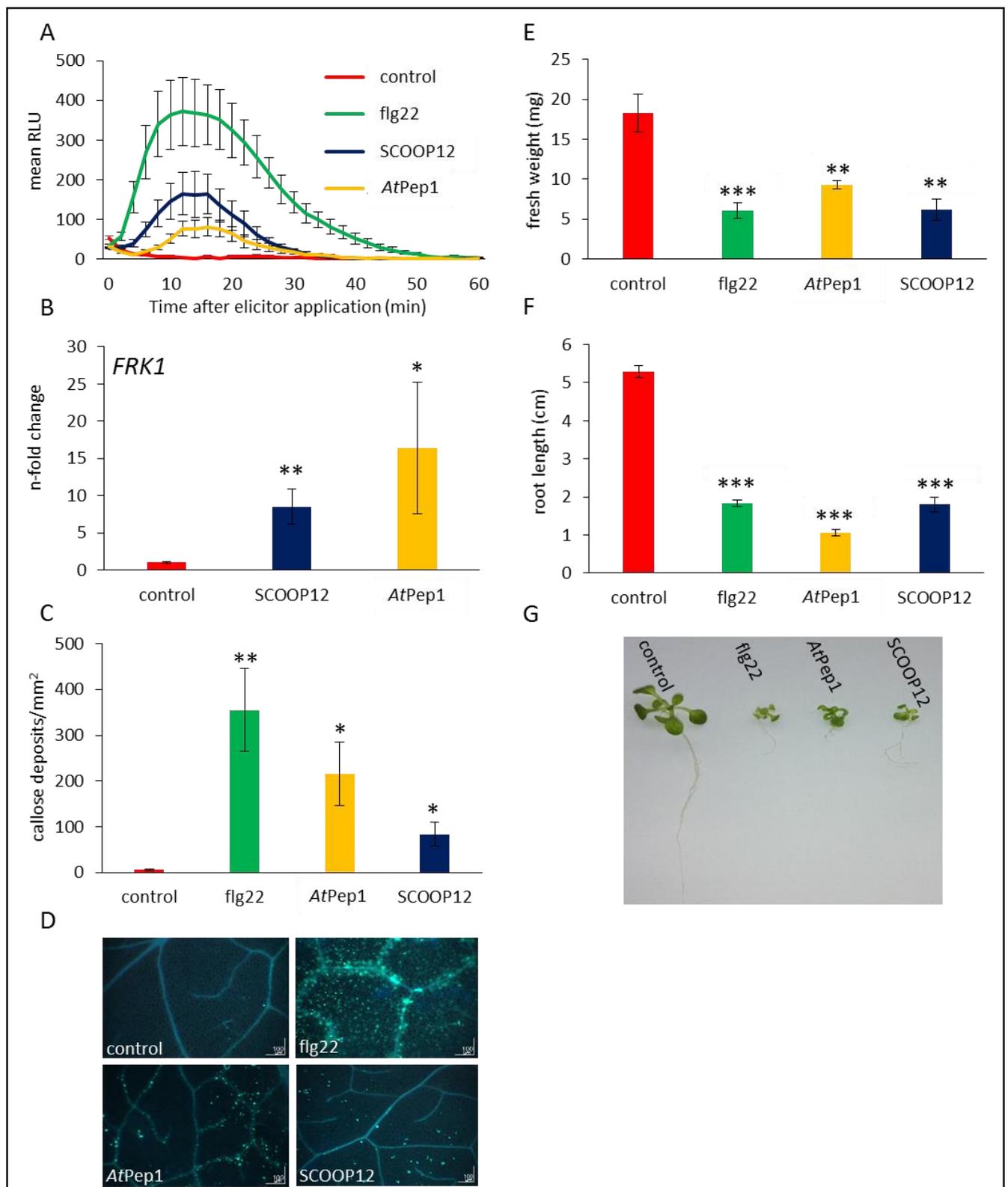


Fig. 6. Defense responses induced by SCOOP12. (A) Reactive oxygen species (ROS) in RLU (relative light units) production in wild-type Arabidopsis leaf-discs (Col-0), treated with 1μM for each peptide or without elicitor (control). Graphs display averages of 12 replicates. Error

bars show the \pm SE of the mean. (B) Induction of *FLG22-INDUCED RECEPTOR-LIKE KINASE1* (*FRK1*) gene transcription in soil-grown plants treated with 1 μ M of the indicated peptide or without elicitor (control). Error bars show the \pm SD of the mean based on three biological replicates. (C) Quantification of callose deposition. Error bars represent the \pm SE of the mean of 4 replicates. (D) Localization of callose deposition by aniline blue staining. (E, F, G) Quantification of seedling growth inhibition. 5 days old seedlings were transferred from solid MS medium to liquid medium supplied with the indicated elicitors (all applied in a final concentration of 1 μ M) and are grown for additional 8 days before fresh weight and root length was quantified and pictures were taken. For all experiments, error bars show \pm SE of the mean of 6 biological replicates. Significant differences according to Student's t-test results: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Figure 7

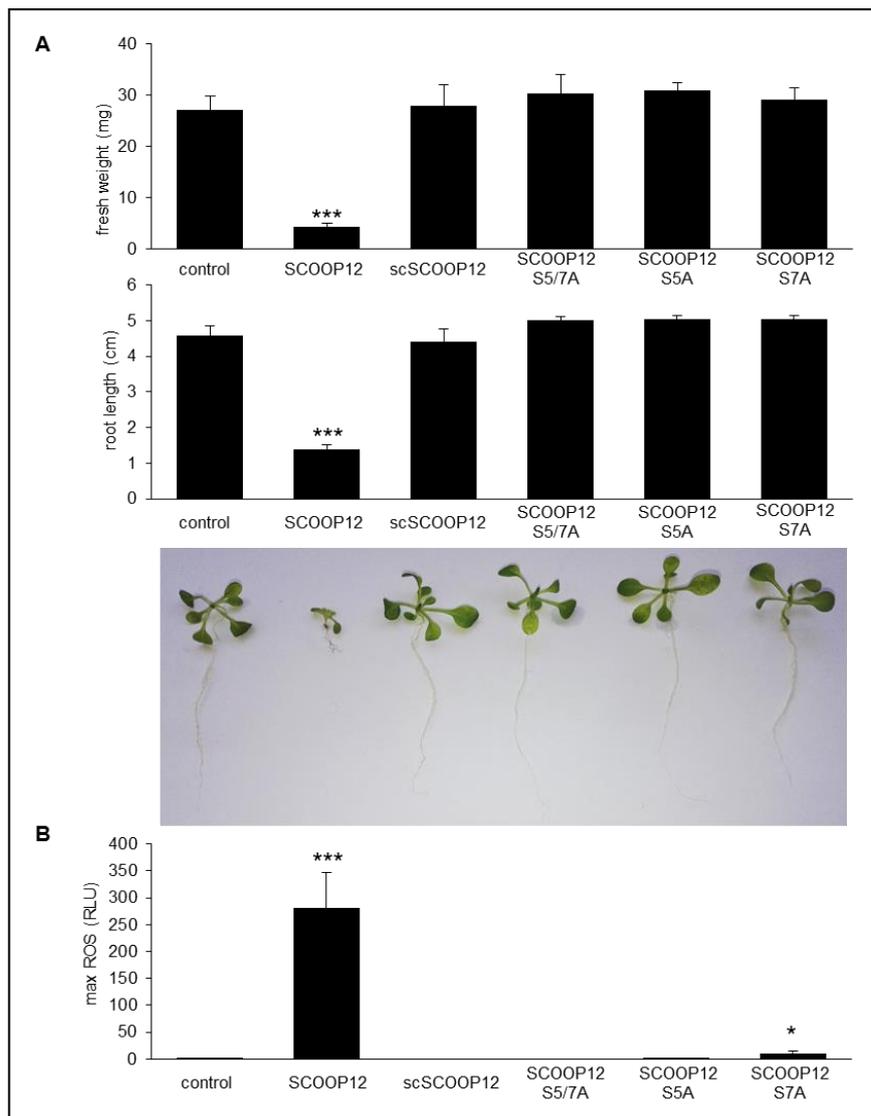


Fig. 7. SCOOP12 activity depends on the correct amino-acid order and two highly-conserved Serine. Assays were carried out with scrambled peptide (scSCOOP12) and alanine replacements of conserved serine residues on position 5 and 7 of SCOOP12 (PVRSSQSSQAGGR) (SCOOP12 S5/7A; SCOOP12 S5A; SCOOP12 S7A). (A) Quantification of seedling growth inhibition with the indicated elicitors. Bars of quantified fresh weight and root length represent mean of six replicates. (B) Reactive oxygen species (ROS) in RLU (relative light units) production in wild-type Arabidopsis leaf-discs (Col-0), treated with 1 μ M for each peptide or without elicitor (control). Graphs display averages of 12 replicates. Error bars show \pm SE of the mean. Significant differences according to Student's t-test results: *, $P < 0.05$; ***, $P < 0.001$.

Figure 8

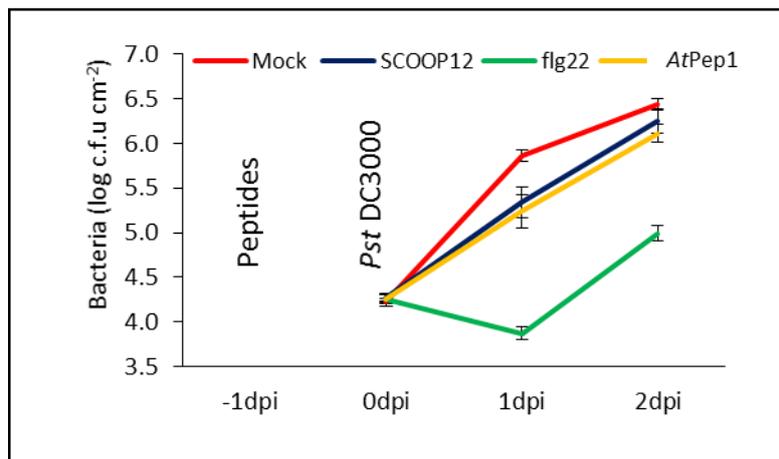


Fig. 8. SCOOP12 application protects against *Pseudomonas* infection. Arabidopsis wild-type (Col-0) plants were pre-treated for 24h by leaf infiltration with 1 μ M of the indicated elicitor or without peptide. Subsequently, leaves were infected with 10⁵ cfu.ml⁻¹ *Pst.* DC3000, and bacterial growth was assessed 1 and 2 days after infection. Plot represents the mean of 8 replicates and error bars show the \pm SE of the mean. Excepted between *AtPep1* and SCOOP12, all differences are statistically significant at 1dpi and 2dpi ($P < 0,05$).

Figure 9

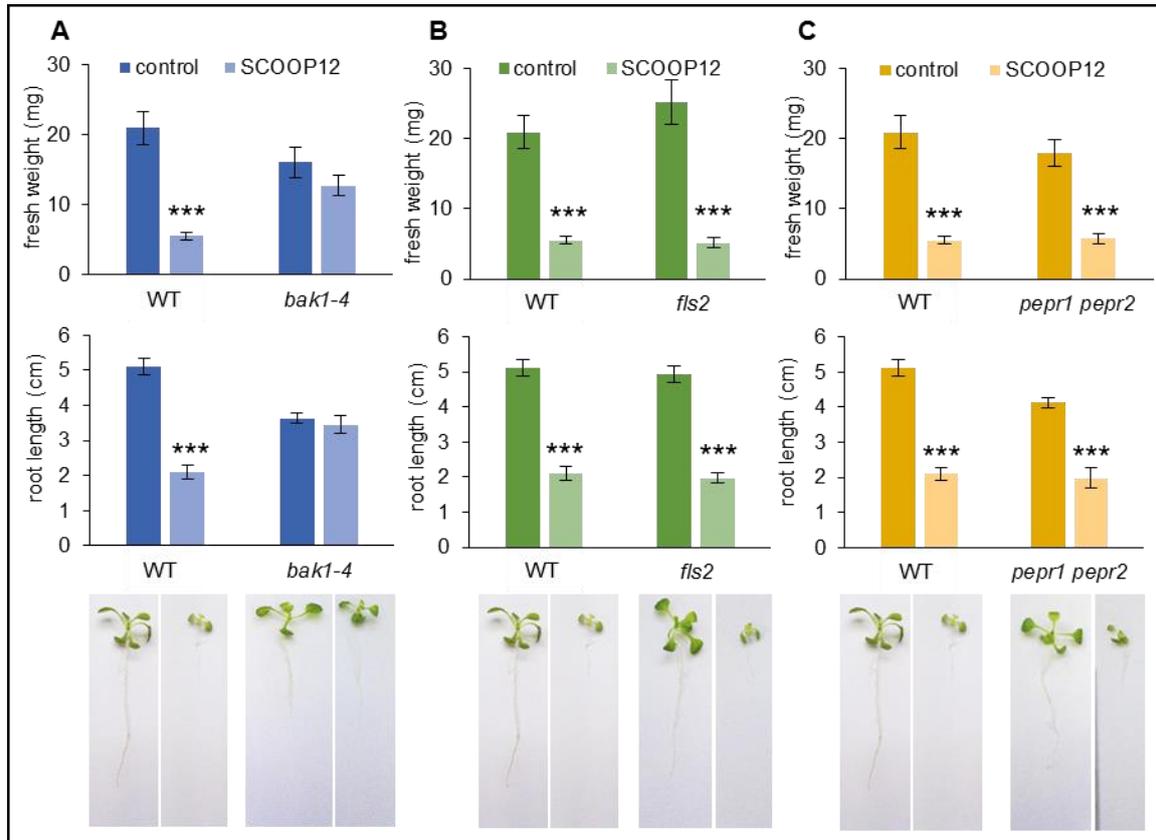


Fig. 9. Seedling growth inhibition assay on selected receptor mutant backgrounds. (A) *bak1* plants were insensitive to SCOOP12. Neither fresh weight (top) nor root length (center) were affected by SCOOP12 treatment. The *FLS2* (B) and *PEPR1/PEPR2* (C) receptor mutants were not affected in their perception of SCOOP12. Plants were grown for 8 days in presence of 1 μ M SCOOP12 or control solution. Bars of quantified fresh weight and root length represent mean of six replicates. Error bars show \pm SE of the mean. Significant differences according to Student's t-test results ***, P < 0.001.

Figure 10

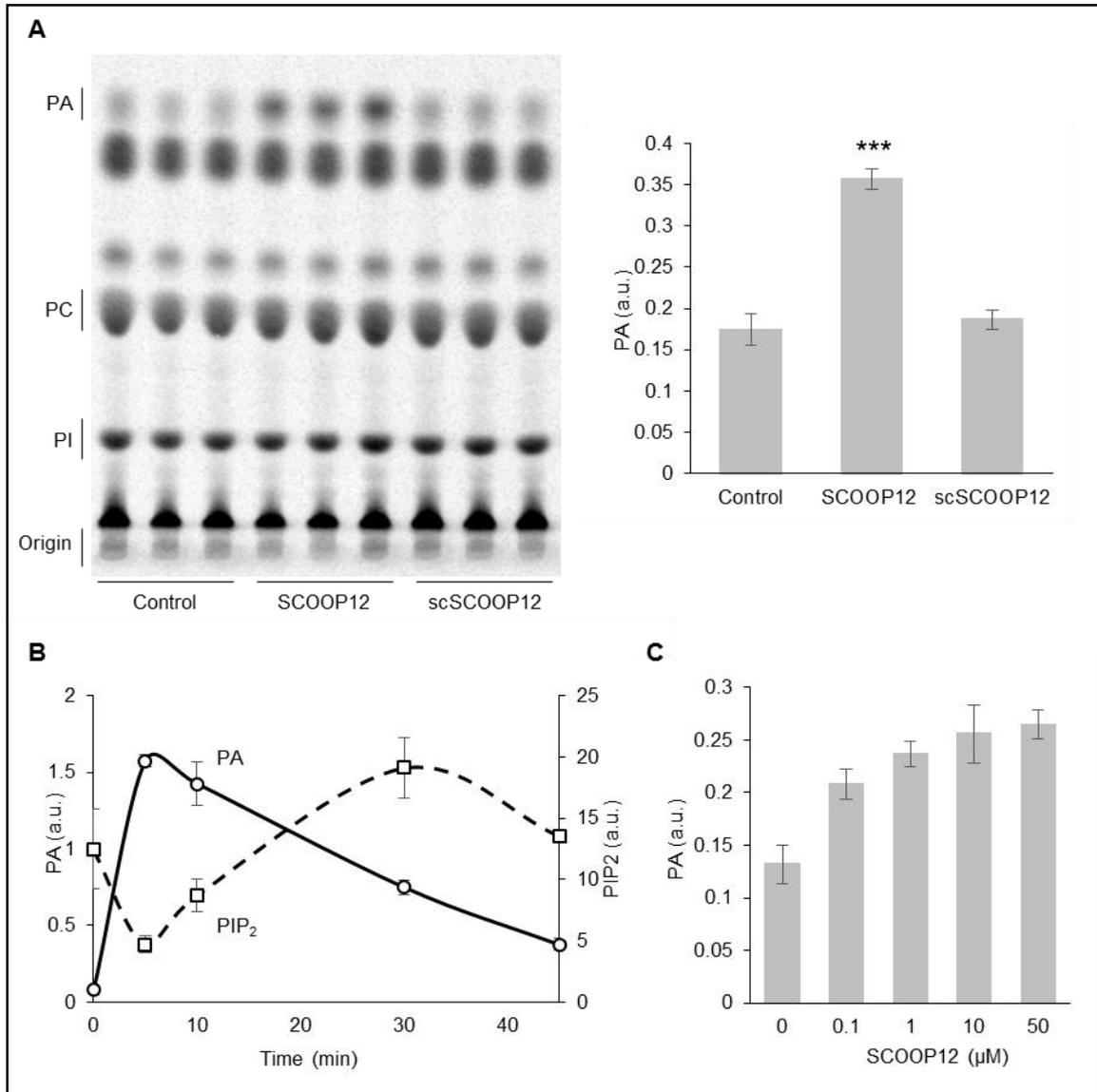


Fig. 10. Rapid activation of PA production in Arabidopsis cell suspensions following treatment with SCOOP12. (A) Separation of P³³-labelled lipids using thin-layer-chromatography with contrasting effects of SCOOP12 (10 μ M) and scrambled scSCOOP12 (10 μ M) on the level of PA accumulation visible after 5 min of treatment. Significant differences according to Student's t-test results: ***, $P < 0.001$. (B) Time-scale of the SCOOP12 (1 μ M) influence on PA and PIP₂ accumulation in Arabidopsis cell suspensions. (C) Dose-scale of the SCOOP12 influence on PA and PIP₂ accumulation in Arabidopsis cell suspensions after 5 min of treatment. All experiments were performed with at least three biological replicates. Error bars show \pm SE of the mean. PA, phosphatidic acid; PIP₂, phosphatidylinositol 4,5-bisphosphate; PI, phosphatidylinositol; PC, phosphatidylcholine; a.u., arbitrary units.

Figure 11

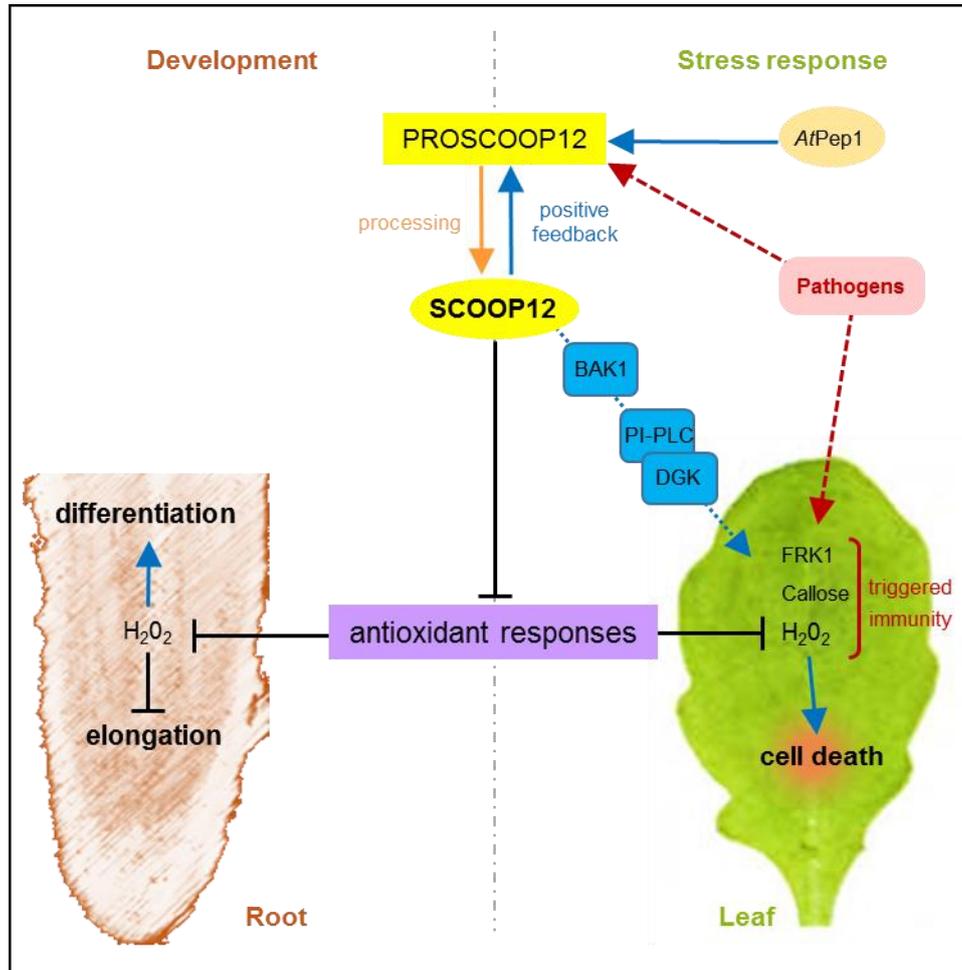


Fig. 11. Putative model explaining the SCOOP12 functions in root development and biotic stress response through the inhibition of protection against oxidative stress. The red dotted arrows represent the action of the pathogens, the induction and the repression effects are represented by blue and black lines respectively. PI-PLC: phosphatidylinositol-specific phospholipase C, DGK: diacylglycerol kinase.

Figure S3

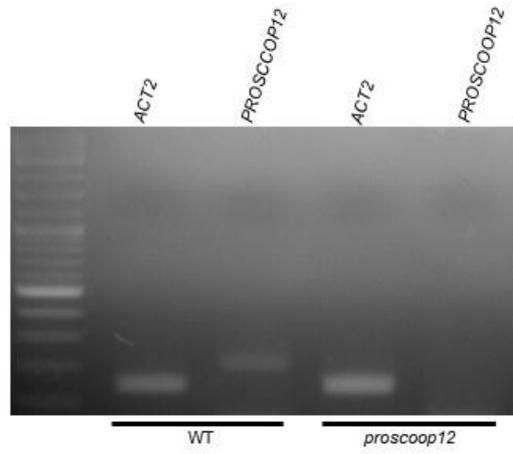


Figure S3. Confirmation of absence of transcription in the *proscop12* T-DNA knock-out line by RT-PCR. *Actine2* expression (*ACT2*) is used as control.

Figure S4

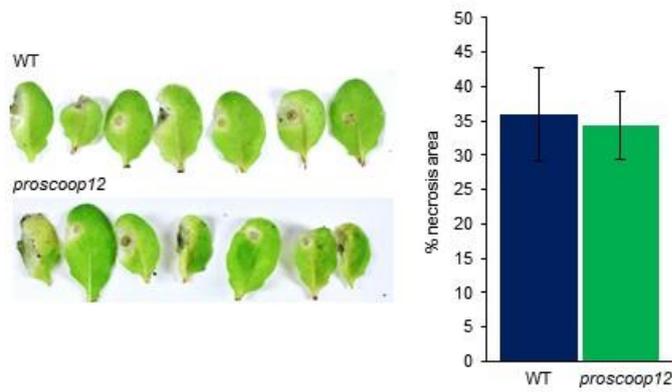


Figure S4. Effect of *A. brassicicola* infection on *proscop12* leaves. The percentage of necrosis area observed six days after fungal infection is not different between *proscop12* and wild-type leaves (Student's t-test, error bars show \pm SE of the mean).

Figure S6

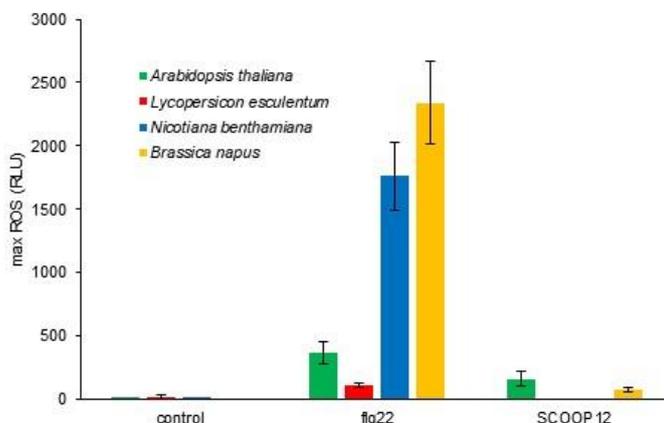


Figure S6. ROS burst measurements on selected plant species treated with SCOOP12. ROS burst assay was performed on *Arabidopsis thaliana* (Col-0), *Lycopersicon esculentum*, *Nicotiana benthamiana* and *Brassica napus*. The flg22 and SCOOP12 peptides were added to a final concentration of 1 μ M. Bars display the average of the maximum ROS burst in RLU (relative light units) of 12 replicates. Error bars show \pm SE of the mean.

Figure S7

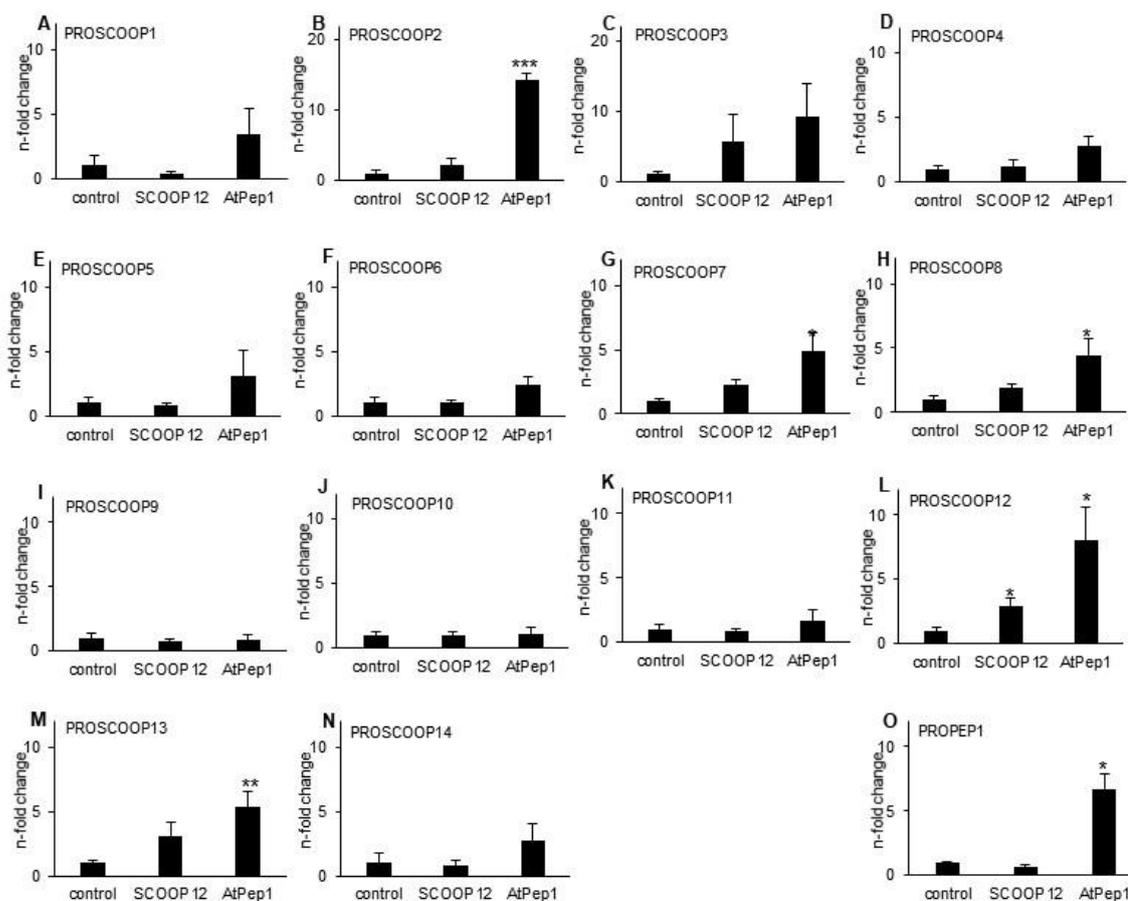


Figure S7. Transcriptional response of the *PROSCOOP* gene family to SCOOP12 and AtPep1. SCOOP12 and AtPep1 induce the expression of several *PROSCOOP* gene family members. Expression level of the *PROSCOOP* gene family members (A to N) as well as *PROPEP1* (O) were determined by normalization to *ACR12* transcripts, and bars indicate the fold change of transcription relative to the control treatment of at least five independent biological replicates. Error bars show the relative \pm SE of the mean. Significant differences according to Student's t-test results: *, P < 0.05; **, P < 0.01; ***, P < 0.001.

11. Curriculum Vitae

(October 2018)

Kay Gully

kay.gully@inra.fr

0033 7 83 49 93 84

49000 Angers

France



PERSONAL

Birth date: 19.06.1988 in Lörrach Germany

Nationality: German

Family status: Unmarried

Languages: German, English and French

CURRENT POSITION

University of Angers, France

Angers, France

PhD student at INRA Angers

10/2015 – present

EDUCATION

Bayer Crop Science fellow

Angers, France

Jeff Schell scholarship for agricultural science

01/2015 – 10/2015

University of Basel, Botanical institute

Basel, Switzerland

Master student

08/2013 – 12/2014

University of Basel, Biocenter

Basel, Switzerland

Bachelor of Biology with mayor in integrative biology

09/2009 – 07/2013

Mathilde Planck high school for biotechnology

Lörrach, Germany

A level student

09/2005 – 08/2008

GRANDS AND AWARDS

Best PhD presentation

10/2017

PhD conference of the “Structure Fédérative de Recherche QUALitéet SAnté du Végétal (SFR QUASAV)”

Best poster price

09/2016

VENAM doctoral school PhD conference

Jeff Schell scholarship for agricultural science by Bayer Crop Science

01/2015 – 10/2015

Project: Epigenetic control of Apple-Pathogen interactions

Supervised by Dr. Etienne Bucher

RESEARCH EXPERIENCE**University of Angers**

Angers, France

PhD student at INRA Angers; Supervisors Dr. Etienne Bucher and Dr. Marie-Noelle Brisset 10/2015 – present

The SCOOP12 peptide regulates defense responses and root development in Arabidopsis

- Characterization of the recently discovered small peptide family SCOOP
- Plant immunity assays including ROS burst assays, seedling growth inhibition assay, MAPK western blot assay, Callose deposition assay, protection assay
- Cloning techniques including PCR, construction of vectors, generation of transgenic plants, gateway cloning, CRISPR-Cas9 approach

Epigenetic regulation of plant defense on Arabidopsis and apple

- Expression determination techniques including qPCR and microarray
- Epigenetic laboratory techniques including Chromatin immunoprecipitation (ChIP)
- Establishing plant immunity assays on apple plants including growth inhibition assay and callose deposition assay on in-vitro grown apple plantlets

University of Basel

Master student in the group of Prof. Dr. Thomas Boller, supervised by Dr. Sebastian Bartels Basel, Switzerland

Perception of AtPeps accelerates starvation-induced senescence

- Effect of DAMPs and MAMPs perception on starvation-induced senescence in Arabidopsis

TEACHING EXPERIENCE

Main assistant in lab practical: “Plant cell and molecular biology” of the university Basel bachelor “Block course”

Basel, Switzerland

09/2014

Assistant in several different laboratory courses: Plant physiology and botanical microbiology, Plant anatomy

Basel, Switzerland

2013/2014

CONFERENCE PRESENTATIONS

Kay Gully and Sandra Pelletier, Marie-Charlotte Guillou, Marina Ferrand, Sophie Aligon, Emilie Vergne, Mathilde Fagard, Philippe Grappin, Jean-Pierre Renou, Etienne Bucher, Sébastien Aubourg. “Regulation of plant development and defense response by SCOOP12, a new small endogenous peptide. Oral presentation at the Plant peptide and receptor conference 2017, Copenhagen, Denmark, September 2017

Kay Gully and Etienne Bucher. “Molecular mechanisms of elicitor-induced epigenetic changes in Apple and *Arabidopsis thaliana*”. Poster presentation at the 2nd Danube Conference on Epigenetics, Budapest, Hungary, October 2016

PUBLICATIONS

Biotic stress induced priming and de-priming of transcriptional memory in Arabidopsis and apple. **Epigenomes. 2018 (under review)**

Kay Gully, Jean-Marc Celton, Alexandre Degrave, Marie-Noelle Brisset, Etienne Bucher

The SCOOP 12 peptide regulates defense response and root development in Arabidopsis thaliana. **Journal of experimental botany. 2018**

Kay Gully^{1‡}, Sandra Pelletier^{1‡}, Marie-Charlotte Guillou¹, Marina Ferrand², Sophie Aligon¹, Igor Pokotylo³, Adrien Perrin¹, Emilie Vergne¹, Mathilde Fagard², Eric Ruelland³, Philippe Grappin¹, Etienne Bucher¹, Jean-Pierre Renou^{1*}, Sébastien Aubourg^{1*}. (‡ These authors contributed equally to this work)

Perception of Arabidopsis AtPep peptides, but not bacterial elicitors, accelerates starvation-induced senescence. **Frontiers in Plant Science. 2015**

Kay Gully, Tim Hander, Thomas Boller and Sebastian Bartels.

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signé par l'étudiant(e) le **23/ 10 / 2018**

Le système immunitaire des plantes - induction, mémoire et désamorçage des réponses de défense par des éliciteurs endogènes, exogènes et synthétiques.

Mots clés : protection de la plante, phytoytokines, épigénétique

Résumé : En tant qu'organismes sessiles, les plantes doivent réagir rapidement et intensément, via des réponses défensives, pour repousser les pathogènes invasifs. Le système immunitaire des plantes peut être déclenché par des molécules élicitrices exogènes ou endogènes. Une autre classe d'éliciteurs, les éliciteurs synthétiques, contient également des composés promouvant une réponse défensive. Dans ce manuscrit, je décris la découverte et caractérisation d'une nouvelle famille de petits peptides endogènes potentiellement sécrétés (PROSCOOP), dont les membres incluent de petits peptides (SCOOP). Je démontre que les SCOOP sont impliqués dans les mécanismes de défense de la plante et le développement racinaire. Une variété de peptides SCOOP induit des réponses défensives de courtes et longues durées.

De plus, des traitements avec le peptide SCOOP12 induisent une résistance à *Pseudomonas syringae* chez *Arabidopsis*. Dans la seconde partie de cette thèse, je démontre que le traitement des plantes avec un éliciteur synthétique peut mener à une mémoire transcriptionnelle à long terme, et que le challenge subséquent des plantes traitées par application d'un éliciteur exogène désactive cette mémoire transcriptionnelle. En conclusion, ma thèse présente (1) la diversité des fonctions que peuvent avoir ces éliciteurs et (2) l'impact sur les systèmes de défense de la plante et ses conséquences sur la mémoire et le développement de la plante.

The plant immune system - induction, memory and de-priming of defense responses by endogenous, exogenous and synthetic elicitors.

Keywords : plant protection, phytoytokines, epigenetics

Abstract : As sessile organism plants have to react quickly and strongly with defense responses to repel any invading pathogen. The plant immune system can be triggered by exogenous or endogenous elicitor molecules. Another class of elicitors are defense promoting compounds which are also known as synthetic elicitors. Here I describe the discovery and characterization of a novel family of potentially secreted small endogenous peptides (PROSCOOP) which members harbor small peptides (SCOOPs). I show that the SCOOP family is involved in plant defense and root development. Various SCOOP peptides induce short- and long-term defense responses. Moreover, treatments with the SCOOP12 peptide induce the resistance against *Pseudomonas syringae* in *Arabidopsis*

. In the second part of this thesis I show that treatments with a synthetic elicitor can lead to long-term transcriptional memory and that subsequent challenging of such plants with an exogenous elicitor reverted this transcriptional memory. In conclusion, my thesis shows (1) how diverse the function of these elicitors can be and (2) the impact the plant defense system and its triggers have on plant development and memory.