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# Identification and study of promoters induced by Asian soybean rust : application in an artificial cell death system

Lisa Cabre

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N°d'ordre



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**Identification and study of promoters induced by Asian soybean rust. Application in an artificial cell death system**

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Devant le jury composé de :

MARCELINO-GUIMARAES Francismar Correa	HDR	(Embrapa, Brazil)	Rapporteure
VOEGELE Ralf	PR, HDR	(Hohenheim University, Germany)	Rapporteur
DUMAS Bernard	DR, HDR	(Université Toulouse III)	Examinateur
PRAT Daniel	PR, HDR	(Université Lyon1)	Examinateur
POUSSEREAU Nathalie	MDC, HDR	(Université Lyon 1)	Directrice de thèse
PELISSIER Bernard	PhD	(Bayer Cropscience Lyon)	Co-encadrant







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Titre : Identification et étude de promoteurs induits par la rouille asiatique du soja. Application à un système de mort cellulaire artificielle.

Mot clés : Soja, *Phakopsora pachyrhizi*, promoteurs inductibles, GFP, mort cellulaire.

RESUME :

*Phakopsora pachyrhizi* Syd. & P. Syd. est le plus important fléau du soja (*Glycine max* (L.) Merrill). Introduit au Brésil dans les années 2000, ce champignon s'est rapidement répandu sur les deux continents Américains. Seule l'utilisation de fongicides associée à des pratiques culturales strictes permet de maintenir le niveau de production. L'utilisation répétitive de ces fongicides ainsi que la plasticité génétique de ce champignon ont rapidement entraîné une diminution d'efficacité de certaines molécules. Par ailleurs, la plupart des résistances verticales identifiées dans les ressources naturelles du soja restent inefficaces contre certains isolats du champignon. La compréhension des mécanismes de l'immunité des plantes permet de proposer des solutions biotechnologiques pour le contrôle des maladies.

L'utilisation antérieure du système barnase/barstar induisant une mort cellulaire artificielle, a permis de générer des pommes de terre résistantes à *Phytophthora infestans*. Cette technologie est basée sur l'expression de la barnase une ribonucléase toxique pour les cellules, et la barstar un inhibiteur de la barnase. Il a été proposé d'évaluer ce système pour le contrôle de *P. pachyrhizi*. Le point critique de cette approche est de trouver le bon rapport de l'expression des gènes barnase/barstar. Pour ce faire la barnase sera placée sous le contrôle d'un

promoteur induit par le pathogène, permettant une régulation spatiotemporelle.

La recherche de tels promoteurs a été effectuée en utilisant des données transcriptomiques et bibliographiques. Des sojas stables exprimant les différentes fusions promoteur:GFP ont été créées afin d'étudier l'expression spatiotemporelle de ces promoteurs en présence du champignon. Les promoteurs pGmCHIT1 (de *G. max*) et pgst1 (de *Solanum tuberosum*) contrôlant respectivement l'expression d'une chitinase et d'une glutathione-S-transférase ont été identifiés comme induits par le pathogène. L'impact de différents stress sur ces deux promoteurs a été évalué.

Les constructions génétiques « barnase/barstar » comprenant les différentes combinaisons des promoteurs ont été générées. *Nicotiana benthamiana* a été utilisé pour exprimer transitoirement les construits et évaluer leur phytotoxicité en absence du pathogène. Un seul construit contenant le promoteur *gst1* s'est avéré non phytotoxique. Il a été transféré avec succès dans le soja. Ces sojas n'ont pas montré de gain de tolérance à la rouille. Une proposition d'amélioration du système barnase/barstar est discutée afin de mieux cerner les possibilités et les limites de ce système pour le contrôle de la rouille du soja.



Title: Identification and study of promoters induced by Asian soybean rust. Application in an artificial cell death system.

Keywords: Soybean, *Phakopsora pachyrhizi*, inducible promoters, GFP, cell death.

## SUMMARY

*Phakopsora pachyrhizi* Syd.& P.Syd, the fungus responsible for Asian soybean rust, is the most devastating soybean (*Glycine max* (L.) Merrill) pathogen. First observed in the 2000s in Latin America, the pathogen has spread throughout the Americas. The control of this pathogen depends on the use of fungicides and strict agricultural practices. The repetitive use of the 3 classes of fungicides and the genome plasticity of the pathogen have led to a decreased efficacy of certain molecules. Although vertical resistance genes have been mapped in the soybean germplasm, most of them are not effective against all Asian soybean rust isolates. A deeper understanding of plant immunity facilitates the development of biotechnological approaches for plant disease control.

Artificial cell death was previously developed to control *Phytophthora infestans* development in potato. The technology was based on a barnase ribonuclease that is highly toxic to the plant cell and that consequently needed to be expressed only in the presence of the pathogen. The lethal expression of barnase was counterbalanced by barstar, a highly specific inhibitor of barnase. We propose to evaluate this technology in soybean to control *P. pachyrhizi*. The key objective is the modulation of the ratio of barnase/barstar based on the identification of

an adequate inducible promoter to control the expression of barnase.

The previous literature and transcriptomic data were used to identify candidate promoters for barnase expression. Stable transgenic soybean expressing the different promoter:GFP fusions were generated to test the spatiotemporal activity of the promoters in the presence of the pathogen. pGmCHIT1 (from *G. max*) and pgst1 (from *Solanum tuberosum*) promoters controlling a chitinase and a glutathione-S-transferase, respectively, were identified as induced by soybean rust. The impacts of different stresses on these promoters were evaluated.

Molecular constructs with different promoters driving the barnase and barstar gene combination were generated. *Nicotiana benthamiana* was used to evaluate construct toxicity in the absence of the pathogen. One single construct containing the promoter pgst1 was shown to be non-phytotoxic. This construct was successfully introduced in soybean plants. The generated soybeans were challenged with rust, but no protection was observed. Based on these results, we discuss how to improve the barnase/barstar system to control soybean rust.



*Phakopsora pachyrhizi* Syd.& P.Syd. est le plus important fléau du soja (*Glycine max* (L.) Merrill) première culture oléagineuse mondiale. Ce champignon phytopathogène introduit au Brésil dans les années 2000, s'est rapidement répandu sur les deux continents Américains de l'Argentine au Canada couvrant la totalité de la zone de production du soja. Seule l'utilisation de fongicides associée à des pratiques culturales strictes permet aux producteurs de soja de maintenir leur niveau de production. L'utilisation répétitive de ces fongicides limités à 3 modes d'action (biosynthèse des stérols, inhibition de la respiration au niveau du complexe II ou du complexe III), l'immense étendue de soja (~34 millions d'hectares pour le seul Brésil) ainsi que la plasticité génétique de ce champignon ont rapidement entraîné une diminution d'efficacité de certaines molécules. Par ailleurs, bien que plusieurs résistances verticales aient été identifiées dans les ressources naturelles du soja cultivé, la plupart sont inefficaces contre certains isolats du champignon rendant l'aspect sélection conventionnelle long et sans garantie de durabilité. Les firmes semencières se sont alors intéressées à l'évaluation d'une approche biotechnologique pour créer et développer des variétés résistantes à ce champignon.

Les mécanismes de l'immunité des plantes et leur sensibilité aux pathogènes sont aujourd'hui compris « dans les grandes lignes » ainsi que les mécanismes biochimiques sous-jacents permettant ainsi de proposer des solutions biotechnologiques pour le contrôle des maladies. La littérature décrit différentes approches qui explorent par exemple la surproduction de protéines impliquées dans la défense des plantes comme les PR (Pathogenesis Related) protéines, ou des technologies plus récentes telles que l'interférence des acides nucléiques. A ce jour, à quelques exceptions près, les résultats sont mitigés, et aucune culture résistante à un pathogène via les biotechnologies n'est commercialisée (hormis la résistance aux virus).

*P. pachyrhizi* est un organisme biotrophe qui nécessite la survie de son hôte pour compléter son cycle. *In vitro*, le champignon émet un tube germinatif, éventuellement un appressorium, puis dégénère. L'immunité dans le cas du soja contaminé par la rouille se traduit par un collapse des cellules attaquées (mort cellulaire) non visible à l'œil nu, ou dans des cas de moindre résistance, par des réactions d'hypersensibilité plus ou moins visibles (Red Brown lésions). Cette mort cellulaire plus ou moins rapide entraîne la mort du champignon.

Ce phénomène de mort cellulaire pourrait être reproduit artificiellement en utilisant un système, ayant déjà permis de générer des pommes de terre résistantes à *Phytophthora infestans*. Ce système repose sur l'expression de deux gènes bactériens. La barnase, hautement toxique pour

la plante doit être exprimée uniquement en présence du champignon. L'expression de la barstar (anti ribonucléase), permet la formation d'un complexe inactivant l'activité de la barnase en absence d'infection. Le système barnase/ barstar a été proposé et évalué dans ce travail pour le contrôle de la rouille du soja. Le point critique de cette approche est la balance de l'expression des gènes barnase/barstar qui doit changer en condition d'infection. Un promoteur constitutif est nécessaire pour l'expression de la barstar, afin de parer à tout bruit de fond de la barnase, et un promoteur induit par la rouille du soja est requis pour l'expression de la barnase permettant l'induction d'une mort cellulaire localisée.

Dans un premier temps la recherche de promoteurs induits par la rouille a été effectuée en utilisant des données transcriptomiques et bibliographiques. Plusieurs gènes de soja précocement induits ont été répertoriés puis sélectionnés pour ne retenir que les plus prometteurs en terme d'expression. Cinq promoteurs candidats ont ainsi été sélectionnés. Des sojas exprimant les différentes fusions promoteur:GFP ont été générés afin d'étudier l'expression spatiotemporelle de ces promoteurs en présence du champignon. Le promoteur de soja *pGmCHIT1* contrôlant l'expression d'une chitinase et le promoteur de pomme de terre (*Solanum tuberosum*) *pgst1* celle d'une glutathione-S-transférase, ont tous les deux montré une induction par *Phakopsora pachyrhizi*. Ces deux promoteurs ont été sélectionnés pour une évaluation plus poussée de l'impact de différents stress (blessures, traitements hormonaux) sur leur activité. Le promoteur *GmCHIT1* a montré être induit localement par une blessure mais pas par traitement avec un précurseur d'éthylène et avec un analogue du méthyl jasmanate. Le promoteur *gst1* est quant à lui induit par un traitement avec un précurseur d'éthylène.

Les constructions génétiques comprenant les différentes combinaisons des promoteurs constitutifs (pNOS et p35S) et inductibles (*pGmCHIT1* et *pgst1*) respectivement sélectionnés pour contrôler l'expression des gènes barstar et barnase ont été générés. Les constructions génétiques « barnase/barstar » ont été transitoirement exprimées dans *Nicotiana benthamiana* afin d'évaluer leur phytotoxicité en absence de pathogène. Un seul construit comprenant la barnase sous contrôle du promoteur *pgst1* s'est avéré non phytotoxique. La phytotoxicité observée sur la majorité des combinaisons évaluées est très certainement dû à une surexpression de la ribonucléase. Elle n'a cependant pas pu être biochimiquement étudiée, limitant ainsi la compréhension du fonctionnement des construits génétiques. Une bonne corrélation entre le modèle *N.benthamiana* et le soja a par la suite été confirmée. Le construit non toxique pour *N.benthamiana* a ainsi été transféré avec succès dans le soja, alors que les construits présentant une phytotoxicité n'ont pas permis d'obtenir de plantes de soja transformées. Seuls les sojas

contenant le système barnase/barstar avec le promoteur *pgst1* ont donc été testés pour leur résistance à la rouille. Ces sojas n'ont pas montré de gain de tolérance avec ce promoteur. Une proposition d'amélioration du système barnase/barstar tenant compte de ces résultats est discutée afin de mieux cerner les possibilités et les limites du système pour le contrôle de la rouille du soja.

Le travail a permis de caractériser un promoteur *pGmCHIT1*, inductible par *Phakopsora pachyrhizi*. C'est à ce jour et à ma connaissance le seul exemple dans la littérature.



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# INTRODUCTION

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## A. GENERAL INTRODUCTION

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In the coming decades, an increasing demand for food and feed is expected to accompany a growing world population. According to the FOOD & AGRICULTURE ORGANIZATION (FAO) (The future of food and agriculture, 2018), the world population will reach 10 billion people by 2050, which is 2 billion more than today. If the current growth of the population continues as suggested, we could exceed 16 billion people at the end of the 22<sup>nd</sup> century (FAO, 2018). Consequently, there will be a need to feed this additional population. According to those predictions, augmentation of crops production is a key challenge.

One strategy to reach this goal is to reduce losses in crop yield. Indeed, abiotic and biotic constraints are responsible for dramatic harvest losses (Ashraf *et al.*, 2012). A recent report showed that more than one hundred fungal pests impact the five main crops, wheat, rice, maize, potato and soybean, causing 20 to 30% annual yield losses worldwide (Savazy, 2019). Additionally, globalization in association with climate change favours plant pathogen epidemics as well as the emergence of infectious diseases (FAO, 2018). One example is the citrus Huanglongding (HLB) that constitutes the most economically devastating disease of citrus worldwide. This disease is caused by *Candidatus Liberibacter* bacterium, transmitted by the psyllids *Trioza erytreae* in Africa and *Diaphorina citri* in Asia and the Americas. HLB is present in around 40 countries and causes die-black, yellow shoots, blotchy mottles on leaves and off-tasting and malformed fruit (Canales *et al.*, 2016). Another example is *Phakopsora pachyrhizi*, the fungal pathogen responsible for the Asian soybean rust disease. This pathogen spread from Asia to the Americas, where it can cause up to 80% soybean yield loss in the most severe cases (Echeveste da Rosa, 2015).

Plant pathogens are difficult to control because their populations are variable in time and space and are able to evolve and disseminate extremely rapidly (Strange and Scott, 2005). Breeding and chemical solutions are the main sources for crop protection. Nevertheless, those solutions can have a limited effective duration due to the high evolutionary potential of pathogens. This situation is well illustrated with *P. pachyrhizi*.

## B. THE SOYBEAN CROP

---

### 1) Soybean production

Soybean (*Glycine max* (L.) Merrill) is a major source of proteins and a valued oilseed crop with applications in both feed and food (Hartman *et al.*, 2011). *Glycine max*, the cultivated soybean, is the descendant of the wild species *Glycine sojae*. From the Fabaceae family, soybean is a diploid organism with 20 chromosomes and a genome of approximately 978 megabases (Mb) (Schmutz *et al.*, 2010). Soybean cultivation was first mentioned in northeastern China almost 5000 years ago. It has become highly important in recent decades, and soybean cultivation has developed all over the world, specifically in the Americas. Soybean consumption has grown particularly quickly in China, where the population has increased dramatically over the last 50 years. In China, soybean is largely used in human nutrition (tofu) and as meal in the swine industry. To manage the increasing global demand for soybean, planted areas and production have increased substantially (Garrett *et al.*, 2013) (Figure 1-a) The total cultivated soybean area rose from 52 million hectares in 1986 to 103 million in 2010 and 123 million in 2017 (FAO source). In Brazil, the cultivated soybean area increased by almost four-fold and soybean production by 9-fold (Figure 1a-b) from 1986 to 2017. Other countries of South America (Argentina and Paraguay) showed a production rise in the same period. Today, global production has reached 348 million metric tons, more than 90% of which comes from the USA and South America (the USA followed by Brazil and Argentina) (Figure 1-b). Consequently, most soybean exportation comes from South and North America. China and, to a lesser extent, the European Union are the main import regions (Figure 2).

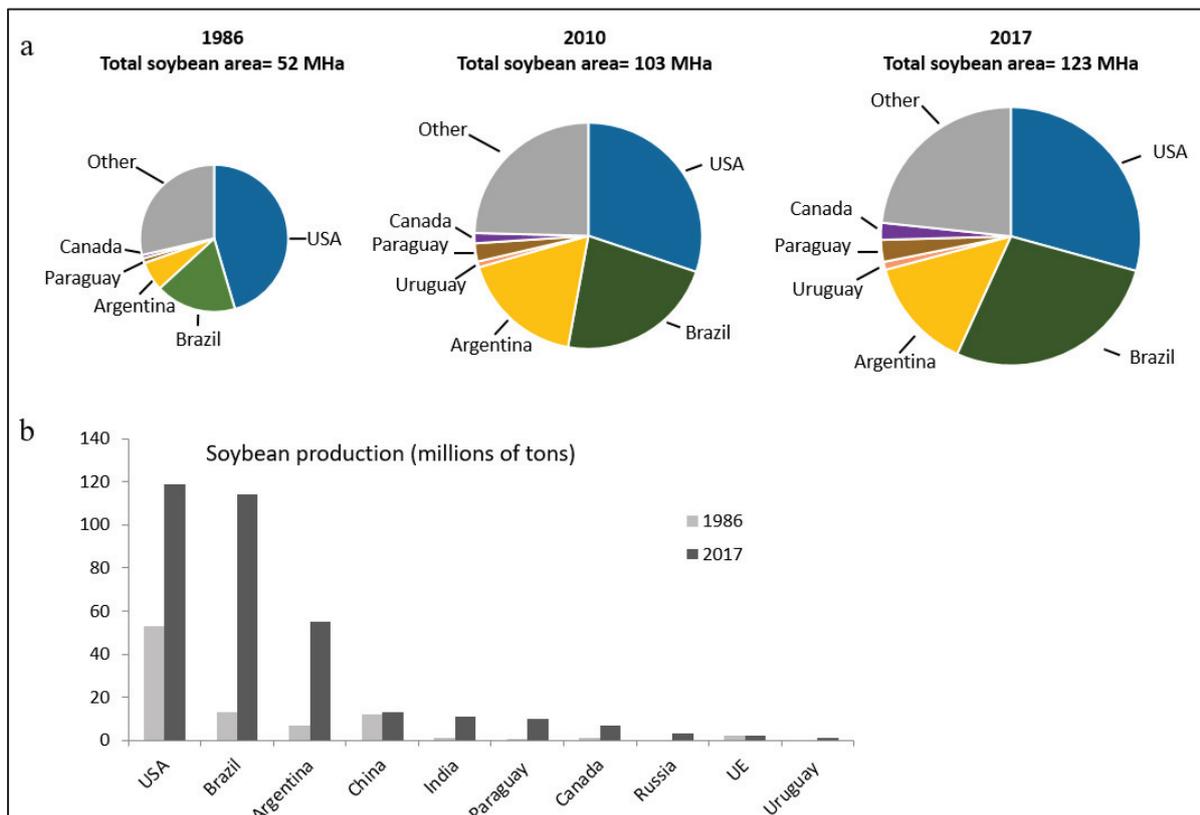


Figure 1: **Increase in soybean planted area and production** a) Soybean cultivated area in 1986, 2010 and 2017 in North and South America. Each slice of the pie represents the percentage of the total soybean area cultivated on the indicated country. b) Soybean production in 1986 and 2017 in different countries of the world. From FAO.

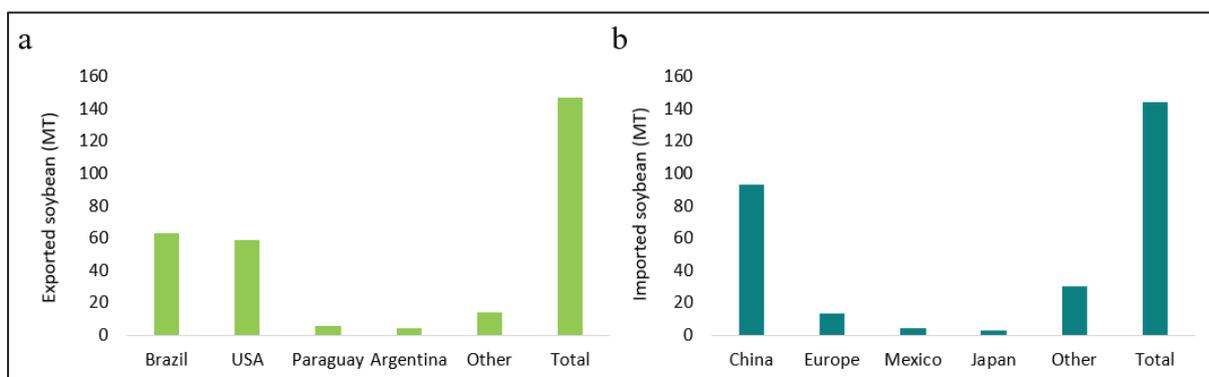


Figure 2: **Soybean import/export**. Millions of tons (MT) of exported a) and imported b) soybean by country. Data for the 2016/2017 season. From USDA.

Soybean grows mainly in warm climates and on a wide range of soils. South and North American climates are appropriate for soybean culture. The growth development period is between 100 and 130 days depending on the latitude. Furthermore, soybean is a legume species able to fix atmospheric nitrogen through symbiotic interaction with soil rhizobia (Stougaard, 2000), limiting the need for N-fertilizers. Nitrogen availability has a major influence on yield and product quality. In the USA, Brazil and Argentina, new rhizobia strains are developed and inoculated onto soybean seeds (Muñoz *et al.*, 2016). Soybean fixation of

atmospheric nitrogen also enhances the chemical, physical and biological properties of soil and helps to improve the yields of the succeeding crops. As a consequence, soybean is often grown as a rotation crop in combination with cotton, maize and sorghum (The soybean: botany, production and uses, 2010).

Cultivated soybeans of North and South America are essentially genetically modified (GM) with herbicide tolerance (Roundup Ready crops, for instance) or lepidopteran protection (“*Bt*” crops). Roundup Ready (RR) crops are tolerant to the herbicide glyphosate. Glyphosate tolerance was obtained through the expression of the 5-enol-pyruvyl-shikimate-3-phosphate synthase (*epsps*) gene from *Agrobacterium* spp. strain CP4. The introduced glyphosate-tolerant EPSPS enzyme meets the plant’s needs for aromatic amino acids and other metabolites that are essential for plant development and growth in the presence of glyphosate (Homrich *et al.*, 2012). “*Bt*” soybeans express a protein (δ-endotoxin) from *Bacillus thuringiensis* (*Bt*) that exerts a toxic effect against lepidopterans. Stacked *Bt*/RR soybeans have been commercialized in Brazil. (Homrich *et al.*, 2012).

The RR varieties have been part of the large extension of soybean cultivation and reflect the widespread acceptance of GM soybean plants among American farmers.

## 2) Importance of soybean for food and feed

Soybean usually contains 40% protein and 20% oil. Consequently, soybeans are an important source of protein and oil for animal and human nutrition (Echeveste da Rosa, 2015). Seeds are crushed to allow oil extraction, and the meal is used as a source of high protein content, mainly for animals (Childs *et al.*, 2018a). Ninety-eight percent of the meal is used for animal feed (livestock and aquaculture), and a very small percentage provides soy flour and protein for human consumption (Hartman *et al.*, 2011) (Figure 3). Eighty-eight percent of extracted oil is used in human food, mostly as cooking oil (Figure 3). A large advantage of soybean is that compared to other crops, it is high in protein and energy (Table 1) (Le Soja Dans les Tropiques: Amélioration et Production, 2010). For instance, 100 g of soybean seeds contain 36.1 g of proteins versus 21 g for the same amount of beef. In Asia, soybean has been a primary source of protein in food due to tofu and soymilk consumption. Today, many such products are available outside Asia and are also consumed for nutritional purposes by people with vegetarian/vegan diets or allergies, for example (Hartman, 2011).

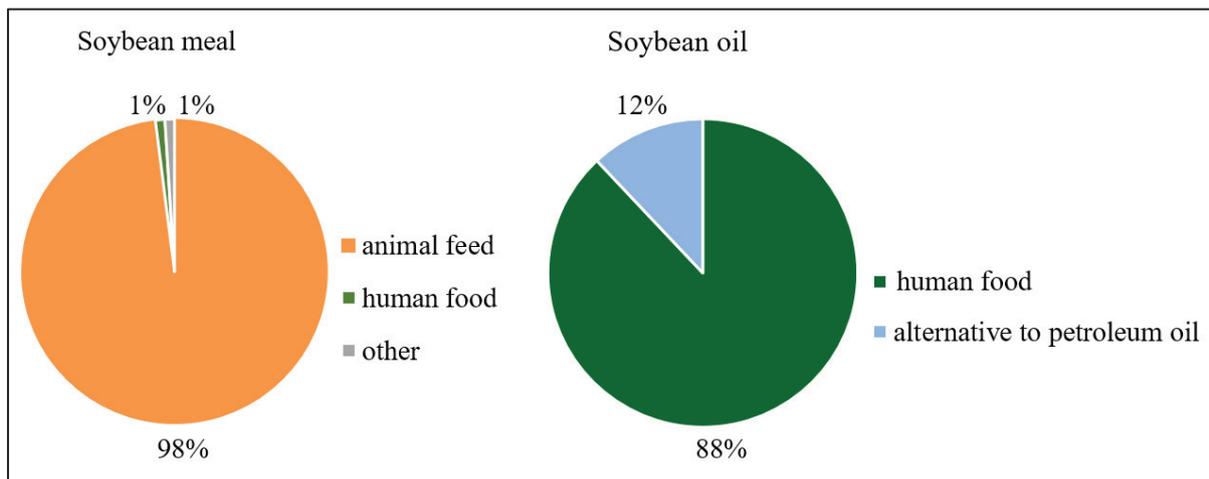


Figure 3: Different uses of soybean meal and oil. Michigan State University (2013).

Table 1: **Composition of raw soybean and other non-transformed foods.** From *Le Soja Dans les Tropiques: Amélioration et Production*, 2010.

Food (100g)	Calories	Proteins (g)	Lipids (g)
Rice	364.0	7.2	0.6
Whole wheat	353.7	12.7	2.5
Corn	363,3	11.8	4.5
<b>Soybean seeds</b>	<b>395.0</b>	<b>36.1</b>	<b>17.7</b>
Beef	111.0	21.0	3.0
Eggs	150.9	12.3	11.3
Cow milk	63.0	3.1	3.5

### 3) Soybean diseases

Soybean is a primary crop for food and feed, and a growing demand for production in the coming years is expected. However, soybean production is impaired in several areas of the world by different foliar and soil diseases. Soybean pathogens and pests can infect/attack all parts of the plant, from roots to seeds (Figure 4).

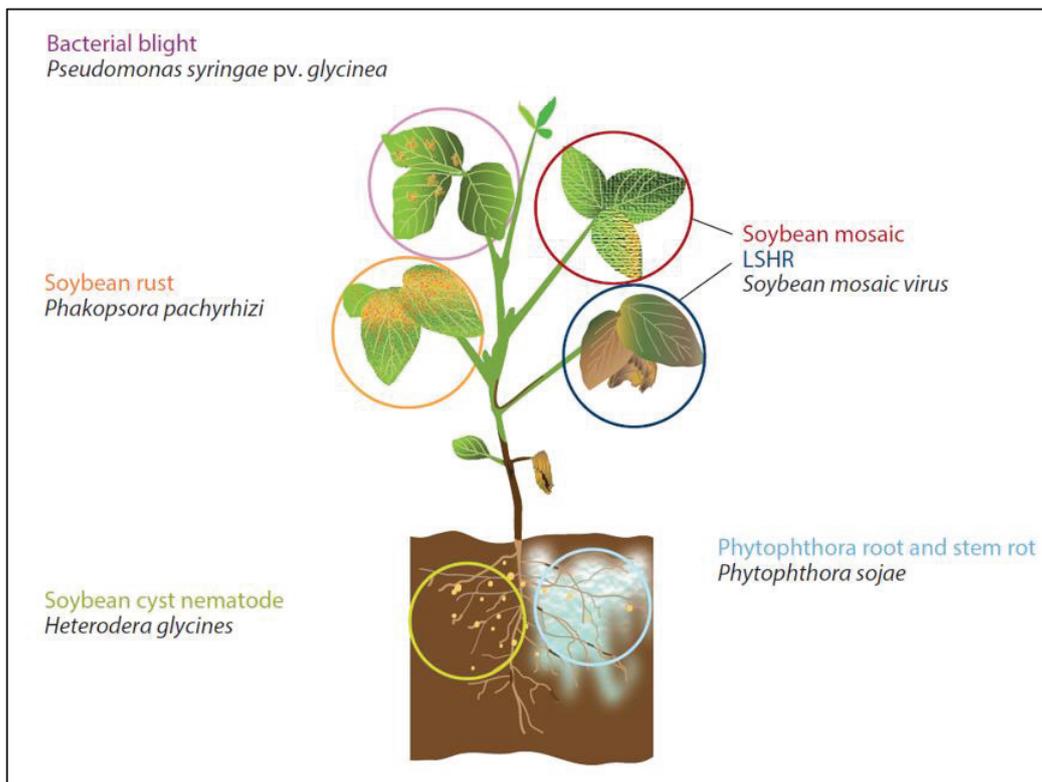


Figure 4: **Examples of soybean pathogens attacking different parts of the crop.** LSHR: lethal systemic hypersensitive response caused by the activation of a hypersensitive response that fails to restrict the virus. Adapted from Whitham *et al.*, 2016.

The soybean cyst nematode (*Heterodera glycines*) causes the soybean cyst disease on roots in most soybean-growing regions. Symptoms on the root system range from slight discoloration to severe necrosis (Whitham *et al.*, 2016). The soybean aphid (*Aphis glycines*), originally found in Asia, has spread to the USA and Canada. This species causes stunting and leaf distortion and reduces pod set. Furthermore, *Aphis glycines* can transmit soybean viruses such as the soybean mosaic virus (Hartman *et al.*, 2011). Bacteria (such as *Pseudomonas syringae* pv. *glycinea* and *Xanthomonas axonopodis* pv. *glycines*) and filamentous eukaryotic pathogens can also infect soybean. The oomycete *Phytophthora sojae* is widespread and can infect seeds, roots, stems, and leaves; in highly susceptible cultivars, it can cause total loss of the crops (Whitham *et al.*, 2016). The fungus *Sclerotinia sclerotiorum* causes wilt and death of entire or portions of plants and is distributed worldwide (Hartman *et al.*, 2011).

*Phakopsora pachyrhizi* is one of the most destructive pathogens of soybean. Therefore, the control of this pathogen is an important goal. In Brazil, where it causes the highest damage, *Phakopsora pachyrhizi* is considered as farmers' number one priority.

## C. ASIAN SOYBEAN RUST DISEASE

---

### 1) Characteristics of rusts

Plant rusts are among the most damaging crop diseases. One characteristic of rust fungi is their high degree of host specificity. A given plant can be resistant to almost all rusts but still serve as a host for a few rust species. For instance, *Puccinia graminis* rust isolates that contaminate wheat are only weakly pathogenic on oat and rye and vice versa (Kolmer *et al.*, 2009). Rust infection is promoted by high humidity during several hours, host plant uniformity and high nutrient availability (Helfer, 2014). Rust fungi are able to evolve and disseminate extremely rapidly. Recently, an evolved race of wheat stem rust (*Puccinia graminis*, strain Ug99) detected in Africa, Asia, and Europe has been reported as a menace to food security due to its ability to overcome resistance in wheat varieties and its potential to spread quickly (Bhattacharya, 2017; Periyannan *et al.*, 2017).

Rust fungi (Uredinales or Pucciniales), parasites of monocotyledon and dicotyledon crops, comprise approximately 7000 species in the Basidiomycota phylum (Kolmer *et al.*, 2009). Fungus-like rusts also exist (mainly white rusts) and are part of the Oomycota phylum. Rust fungi are obligate biotrophic organisms that require a living host to grow and to carry out their reproductive cycle within (Kolmer *et al.*, 2009).

Most rust fungi exhibit a complex life cycle with up to five different spore stages (teliospore, basidiospore, pycniospore, aeciospore and urediniospore) that can occur in two different hosts (telial and aecial host for teliospore and aeciospore formation, respectively). Teliospores produced the previous growing season on the telial host plant, usually on dead host tissues, start to germinate at the beginning of the growing season (Figure 5-a). The diploid teliospores germinate in place and undergo meiosis to produce haploid basidiospores (Figure 5-b), which are ejected into the air. Basidiospores cannot infect the telial host species and must travel to another host (aecial host), where they infect host cells directly through the cell walls. Basidiospores are fragile and do not travel long distances. Basidiospores invade host tissues to produce haploid structures called pycnia. Within each pycnium are produced pycniospores embedded in insect-attracting nectar on the host's surface – leaves, petioles, woody stems or flower parts. Insects or surface moisture (rain or dew) distribute pycniospores on the host surface or between neighbouring plants, resulting in fertilization (Figure 5-c). Once this occurs, the fungus forms the next spore stage (Figure 5-d), the

aeciospore. For rust species that infect leaves, aeciospore formation usually occurs on the lower leaf surface. The colour of the aecial mass is often light orange. Aeciospores are produced in quantity and can travel long distances. This capability is necessary considering that they need to find another host. Once deposited on suitable telial host tissues, usually leaves, aeciospores germinate rapidly and usually invade cells through stomata. The resultant infection results in another pustule (uredinia), which contains urediniospores (Figure 5-e). This stage is capable of repeating on the same host, causing an epidemic. Late in the growing season, when plant derived nutrients are declining, uredinia convert to telia (Figure 5-f) and produce increasing numbers of teliospores (Kolmer *et al.*, 2009).

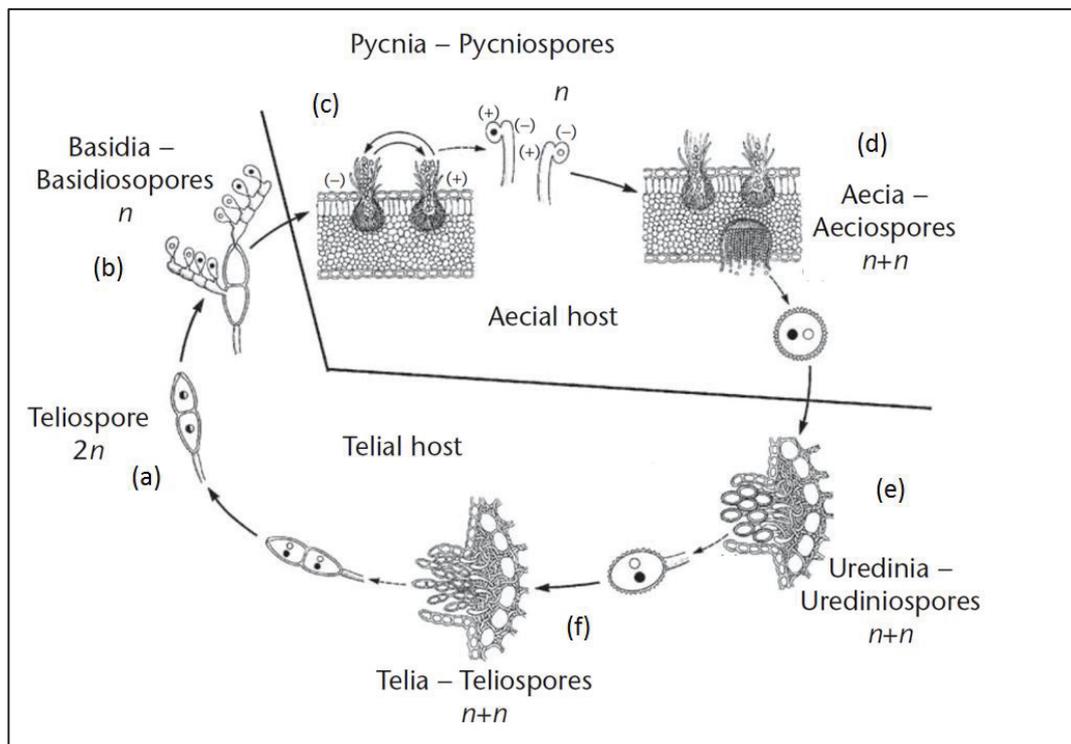


Figure 5: **Common life cycle of rust fungus.** a) Telia with teliospores, b) basidiospores produced by basidia, c) pycnial stage, d) aecia containing the aeciospores, e) uredinial stage that can be reproduced on the same host and f) telial stage.  $n+n$  = haploid state and  $2n$  = diploid state. From Kolmer *et al.*, 2009.

## 2) *Phakopsora pachyrhizi*

*Phakopsora pachyrhizi* Syd. & P. Syd is the fungal pathogen responsible for Asian soybean rust, the most destructive foliar disease of soybean (Echeveste da Rosa, 2015). This fungus is part of the Basidiomycetes class, the Uridinales order and the Phakopsoraceae family (Figure 6) (Stone *et al.*, 2010). The *Phakopsora* genus is separated into two species, *P. pachyrhizi* and *P. meibomiaae*, based on morphological differences and nucleotide sequence analysis. The *P. pachyrhizi* isolates are from the Eastern Hemisphere populations (before it spread), while the *P. meibomiaae* isolates

are only known from the Western Hemisphere and are endemic in Brazil. Both species are able to infect soybean, but *P. meibomia* infection has an inconsequential effect on yield and *P. pachyrhizi* is by far the most virulent species (Bonde *et al.*, 2006).

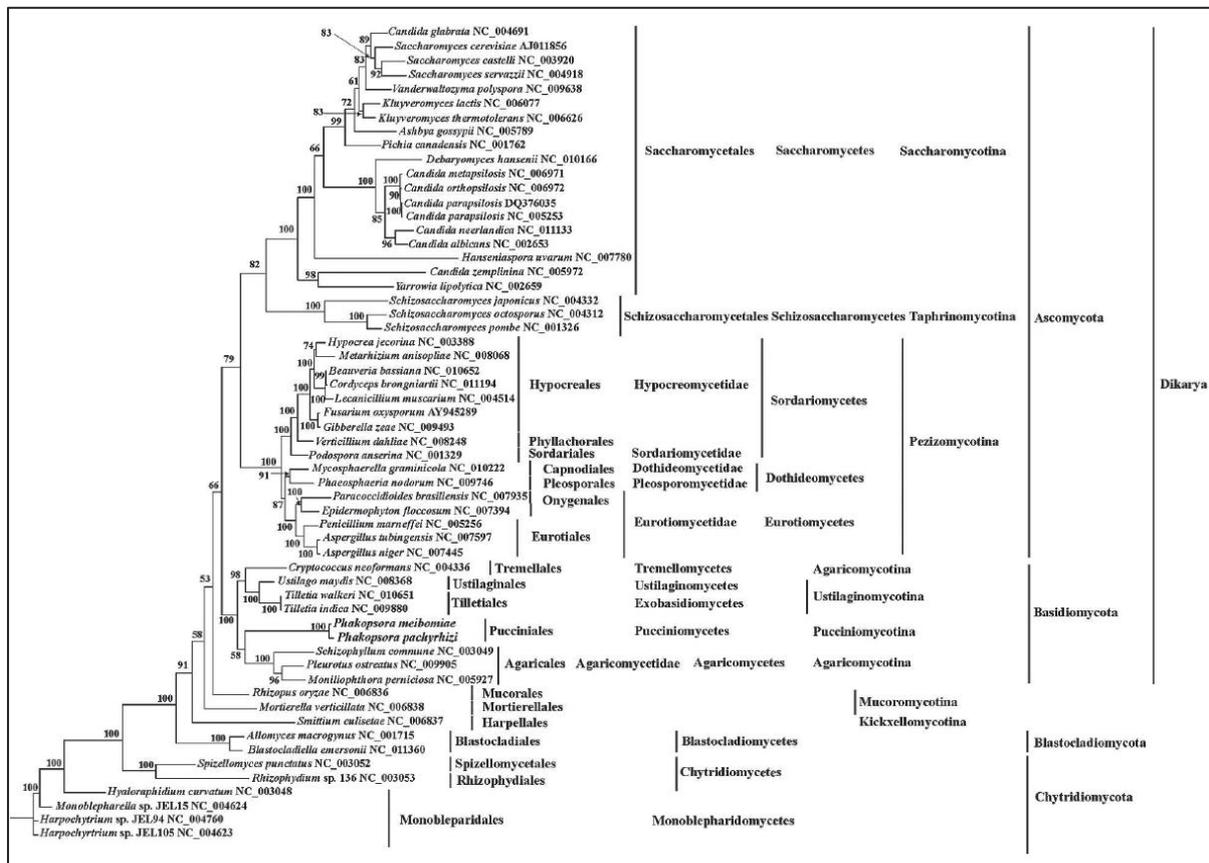


Figure 6: **Phylogenetic relationships among *P. pachyrhizi*, *P. meibomia* and other fungi.** The phylogenetic tree was built with concatenated protein-coding sequences of 14 essential mitochondrial genes (atp6, atp8, atp9, cox1, cox2, cox3, cob, nad1, nad2, nad3, nad4, nad4L, nad5 and nad6) common to 58 fungal species. The topology shown was inferred with PhyML and the JTT model of protein evolution; maximum likelihood-bootstrap support was calculated from 100 replicates. (Stone *et al.*, 2010)

### **Infection process**

The *P. pachyrhizi* life cycle is typical of the majority of rusts and is well described (Figure 7). Soybean infection begins with urediniospore germination on the host leaf followed by germ tube formation (Figure 7 a-b). The optimum temperature for urediniospore germination is between 12°C and 27°C. Urediniospore germination is greater in darkness because light can inhibit or delay the germination process (Koch and Hoppe, 1987). A period of leaf wetness is also necessary and can be approximately 6 h within the optimal temperature range. Approximately 6 h post-contamination, an appressorium is formed (Figure 7-c). Unlike most rusts that penetrate their host via the stomata, *P. pachyrhizi* has the ability to penetrate directly in the epidermal cells of its host. The Asian soybean rust uses mechanical force together with enzymatic digestion to penetrate the host cuticle and cell wall (Edwards and Bonde, 2011) (Figure 8). This process leads to the death

of the penetrated epidermal cells (Figure 7-d) (Keogh *et al.*, 1980). Once in the plant mesophyll, the primary hypha grows through the epidermal cells and reaches the intercellular space of the mesophyll cells (Loehrer and Schaffrath, 2011). Then, a feeding structure, the haustorium, is formed and proliferation of secondary hyphae occurs rapidly approximately 12/24 h post-infection (Figure 7 e-f). The leaf is rapidly invaded with fungal mycelium. Approximately 5-8 days post-infection, uredinia appear on the abaxial sides of the leaves (Figure 7-g). The optimum temperature for uredinia formation is reported to be 17°C at night and 27°C during the day (Kochman, 1979). New urediniospores are released 2-3 days after uredinia formation (Figure 7-h), and contamination of healthy plants occurs through spore dissemination (Goellner *et al.*, 2010). It has been reported that the pathogen is able to produce only two types of spores: the urediniospores (asexual spores) and the avirulent teliospores (necessary for the survival stage of the life cycle). Basidiospores can be observed under controlled conditions (Goellner *et al.*, 2010), but the aecial stage has never been reported in nature (Echeveste da Rosa, 2015).

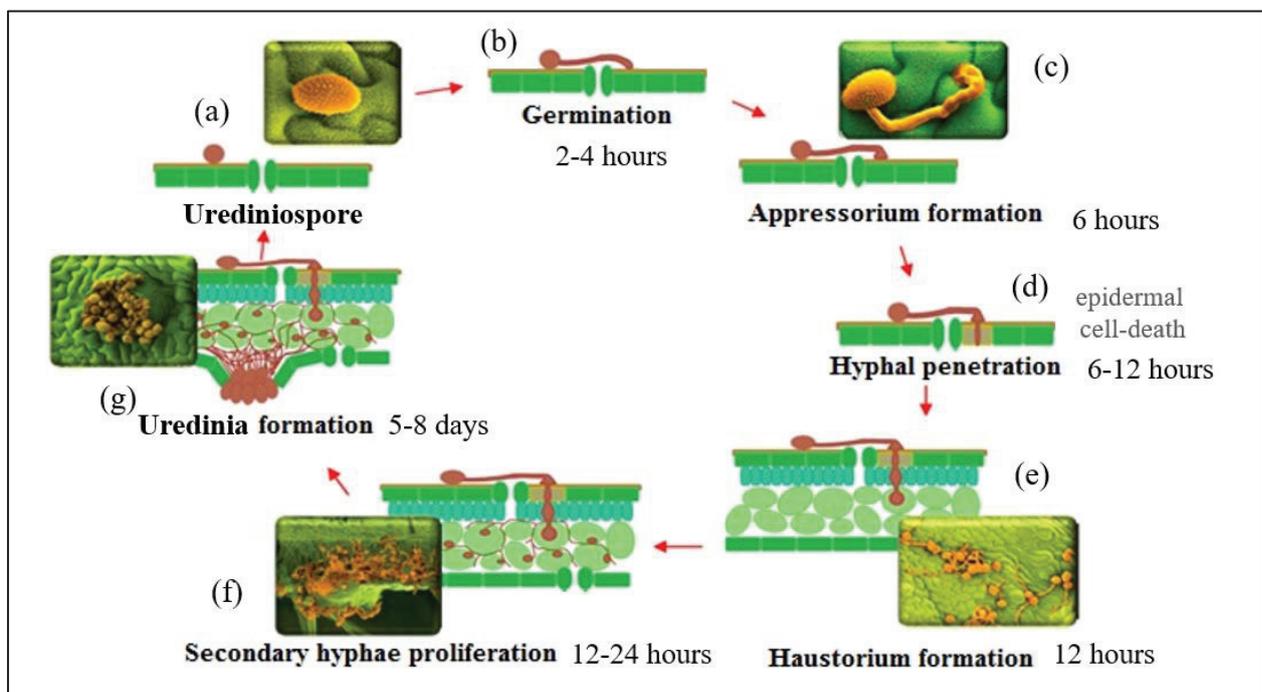


Figure 7: *Phakopsora pachyrhizi* infection cycle. a) A urediniospore on the host plant leaf. b) Urediniospore germination and germinative tube formation followed by c) appressorium formation. d) Penetration of the primary hypha directly in an epidermal cell. The penetrated cell dies, and haustorium development occurs e). The mesophyll is colonized by secondary hyphae, g) and a uredia is formed. After uredia sporulation, new urediniospores will be disseminated. From Goellner *et al.*, 2010; drawings C. Sirven, pictures BayerCropScience.

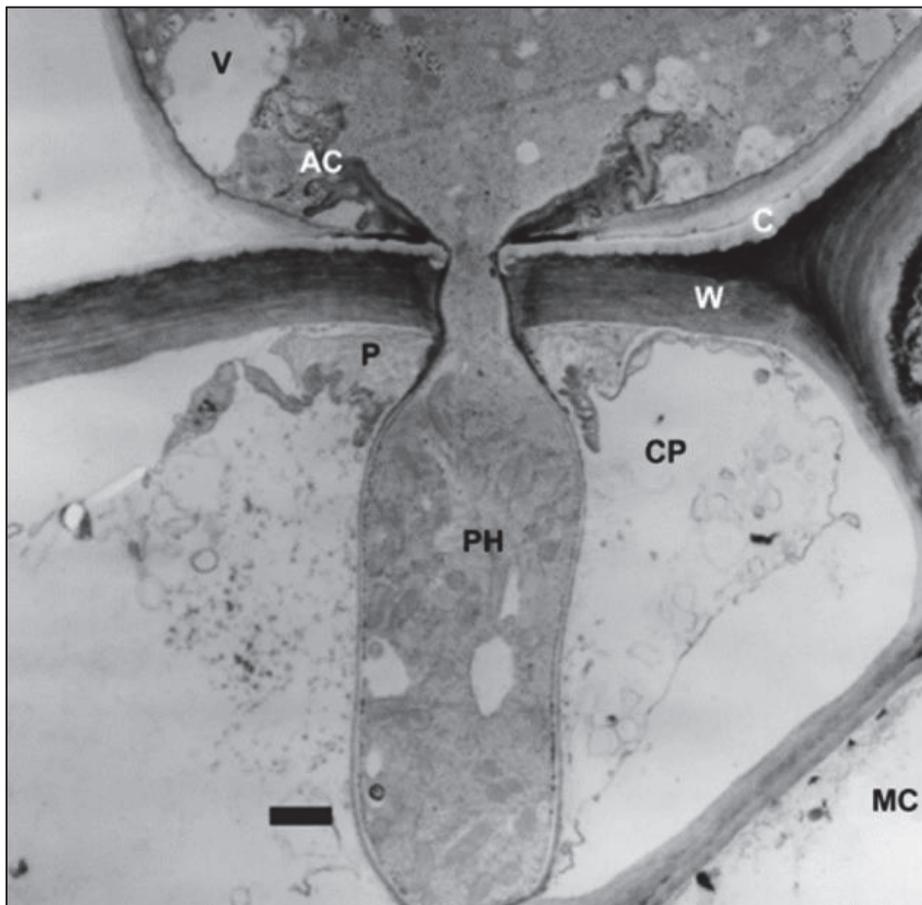


Figure 8: *P. pachyrhizi* penetration of soybean leaf. Penetration hypha traversing a soybean epidermal cell. The infolded cuticle indicates deflection by pressure, and the lack of stress lines in the epidermal wall indicates digestion. AC = appressorial cone, C = cuticle, CP = cytoplasm, MC = mesophyll cell, P = papilla, PH = penetration hypha, V = vacuole, W = host wall. Bars = 1  $\mu$ m. From Edwards and Bonde, 2011.

### Disease symptoms

*P. pachyrhizi* is responsible for leaf disease. Symptoms are characterized by chlorosis of the tissues and punctual lesions visible 4-5 days post-infection (dpi) (Figure 9). Later (6-8 dpi), pustules appear, primarily on the abaxial faces of the leaves. These pustules are the result of uredinia formation, and they produce new urediniospores. Chlorosis develops in most instances around the lesions, with a grey spot in the center of the lesions. Within 12-15 days, the type of necrosis evolves as “tan” colored lesion (TAN). Disease assessment can be realized by estimating the contaminated area on the infected leaves (Godoy *et al.*, 2006). If the disease severity reaches 80%, leaf drop occurs (Echeveste da Rosa, 2015). In consequence, the most severe case of infection can lead to soybean defoliation in the span of only a few days (Figures c-d) as well as rapid plant maturation (Hartman *et al.*, 2005). In this scenario, seed size and weight, oil content and consequently total yield will be reduced (Kumudini *et al.*, 2008).

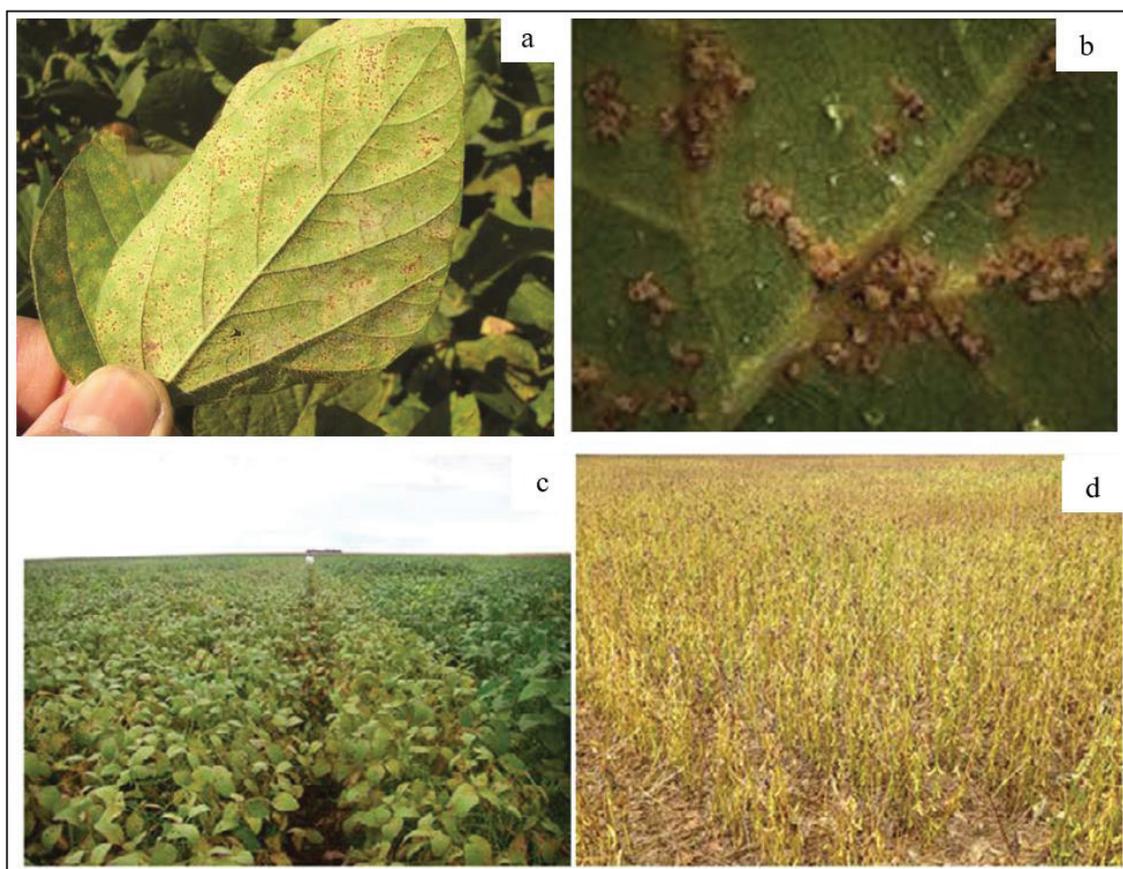


Figure 9: **Soybean rust symptoms and its impact on a soybean field** a) A leaf from a field in Brazil highly infected by *P. pachyrhizi*. From Li *et al.*, 2010. b) A TAN lesion. c) Soybean field highly infected by *P. pachyrhizi* and d) the same field 7 days later, severely defoliated. Location of the pictures: Mato Grosso State, Brazil. From Echeveste da Rosa *et al.*, 2015.

### 3) Impact of *P. pachyrhizi* on soybean crop production

*P. pachyrhizi* was first identified in Japan in 1902 as a pathogen of yam bean (*Pachyrhizus ahipa*) (Li *et al.*, 2010) (Figure 10). The climatic conditions for soybean cultivation being favourable to the development of soybean rust, the pathogen was quickly reported on soybean in Japan. It has been identified throughout the Eastern Hemisphere in Australia, China, India and other soybean-producing countries in tropical and sub-tropical regions (Hartman, 2011) (Figure 10). *P. pachyrhizi* was observed in Africa, where it spread to central, western and southern regions in the mid-1990s (Hartman, 2011) (Figure 10). The pathogen was first reported in the Western Hemisphere in 1994 at Hawaii. The South American continent was free of *P. pachyrhizi* until 2001, when it was reported in Paraguay, near the Brazilian border, resulting in wind dissemination of the fungus into neighbouring countries. Rapidly, the pathogen became established in Bolivia, Brazil and Argentina (Yorinori *et al.*, 2005) (Figure 10). In North America, Asian soybean rust appeared for the first time in the USA in November 2004 as a result of spore propagation by a tropical storm (AgBio Communication Unit, 2005; Schneider *et al.*, 2005).

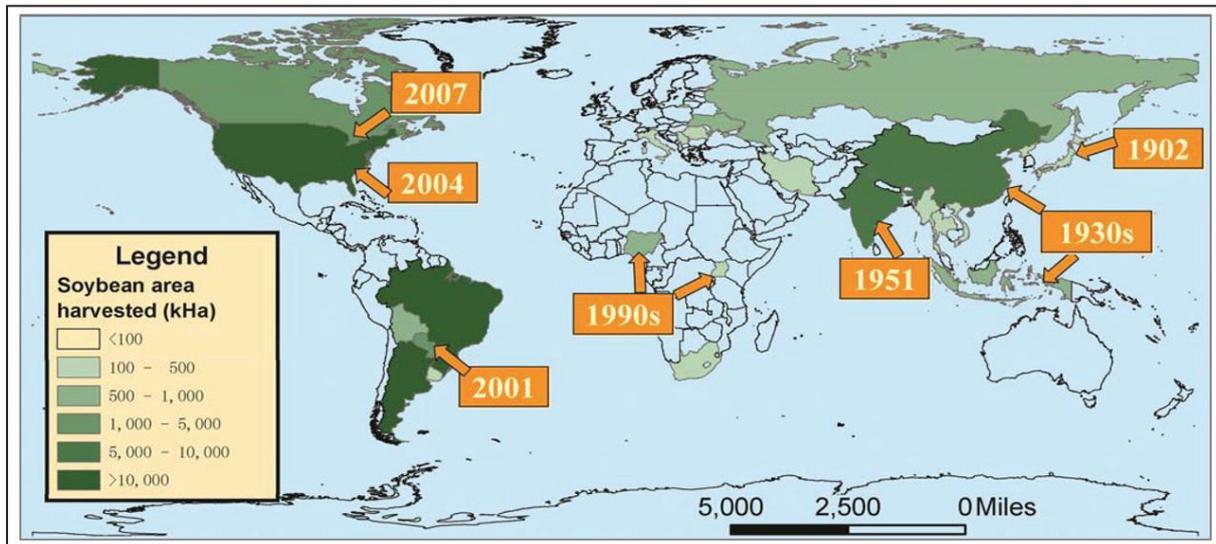


Figure 10: Historical reports of soybean rust and the major soybean-producing countries in 2007. *P. pachyrhizi*. From Li *et al.*, 2010.

By reaching the countries where soybean culture is of high importance, this disease clearly threatens soybean cropping and production. In Brazil, it represents the greatest challenge in the history of soybean production. An example of the threat can be seen in the consequence of the Asian soybean rust epidemic in this country from 2001 to 2004, the first years of its presence in the area. In the 2001/2002 season, rust was detected in 60% of soybean acreage and the total grain losses reached 569 thousand tons (2% of annual production; FAO) (Yorinori *et al.*, 2005). In 2002/2003, the pathogen spread almost throughout the Brazilian territory, and the disease was the most severe where it had not previously been recorded. Total grain losses were estimated at 3.4 million tons, almost 7% of the annual production (Yorinori *et al.*, 2005; FAO). Because the farmers were unaware of the existence of the fungus and lacked knowledge on the appropriate strategy to apply fungicides (preventive instead of curative), total grain losses reached 4.6 million tons in the 2003/2004 season, *i.e.*, 10% of the total production (Godoy *et al.*, 2016; FAO). In 2004, an anti-rust consortium was established to obtain and communicate knowledge of the disease dynamics. In the following years, yield losses decreased as a result of the use of appropriate fungicides and timing of application (Godoy *et al.*, 2016). Consequently, although more expensive, the production of soybean grains in Brazil remained possible due to the protection of the crop with fungicides since the appearance of the rust.

In Argentina, the pathogen is of less importance in the main cultivation zone (Pampa) and only important in the northern areas closer to Paraguay.

In North America, climate conditions being unfavourable for the pathogen, *P. pachyrhizi* does not have a significant impact on soybean production except in the southern states.

#### 4) The success story of *P. pachyrhizi*

The diversity of the Asian soybean rust hosts (non-soybean host and the soybean volunteers disseminated during soybean culture), climatic conditions (warm winter) and its genetic flexibility are the major components of the disease's success in certain areas of Latin America, where soybean is cultivated on a large surface (57 million hectares in total, including 34 in Brazil). Unlike most other rust fungi, *P. pachyrhizi* possesses a wide host range even if restricted to the members of the subfamily Papilionideae of the Fabaceae (Hartman, 2011). The pathogen can infect 31 species in 17 genera of legumes (Ono *et al.*, 1992). The main hosts are *Glycine max*, *G. sojae*, *Pachyrhizus erosus*, *Pueraria lobata* and *Vigna unguiculata*. Under greenhouse conditions, the fungus is able to infect even more species (65 new hosts identified by Slaminko *et al.*, 2008). Alternative hosts of the pathogen such as kudzu (*Pueraria lobata*), an invasive perennial vine, (Figure 11-a) allow the expansion of Asian soybean rust in new geographic areas and represent a source of contamination even when the annual soybean is not cultivated. Therefore, these alternative hosts play the role of “bridge” or intermediary hosts and are a source of fresh inoculum during the following planting soybean season (Figure 11-b) (Hartman *et al.*, 2011).

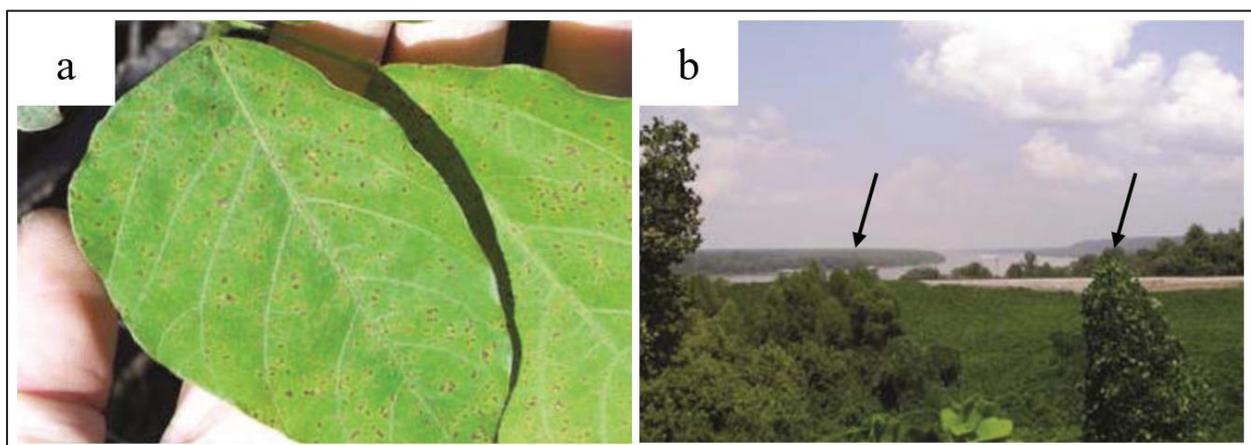


Figure 11: *P. pachyrhizi* on kudzu. Young kudzu leaflets with severe soybean rust infections found in Alabama. b) A large kudzu patch (arrows) in Natchez, MI. From Li *et al.*, 2010.

The climatic conditions are an important factor affecting the disease spread. Indeed, in South America, relatively warm winters (average temperature of 20 °C, Li *et al.*, 2010) are favourable for soybean rust infection at the next planting. The pathogen can remain present year-round, even when the soybean is not cultivated, on soybean volunteers and plants such as kudzu throughout Brazil and in some areas in Paraguay. This capability favours the occurrence of the soybean rust disease every year.

In the Gulf Coast states in the USA, the fungus is also present and can survive throughout the year due to the warm winters. As a consequence, due to spore dissemination, the disease is also reported in the rest of the USA and in Canada. However, Asian soybean rust does not survive to winter in these regions due to colder temperatures. As in Brazil, kudzu has been reported in most of the states of the USA where soybean production occurs, but it has been described as a primary host for Asian soybean rust during winter only in southeastern states (Alabama, Florida, Louisiana, Mississippi and Texas) (Allen *et al.*, 2014). A major collaborative effort has been undertaken to monitor the spread and distribution of the fungus through early detection of the pathogen and notification of the infected areas on public databases in the USA (Sikora *et al.*, 2014). To date, Asian soybean rust has had no impact on soybean yield losses in the USA. However, climate change may increase the temperatures during the winter months, and the isolated area of kudzu infected by the rust could become increase, increasing the threat of this pathogen to the US soybean industry (Sikora, 2014).

The early control of this disease appears to be more important, and different approaches to fighting this pathogen have been and are still being developed.

## 5) Asian soybean rust control

### Fungicides

Today, the control of *P. pachyrhizi* is dependent on the use of fungicides. Demethylation inhibitors (DMIs) impairing sterol biosynthesis, quinone outside inhibitors (QoIs) as well as succinate dehydrogenase inhibitors (SDHIs) that are inhibitors of mitochondrial respiration are the most commonly used fungicides (Langenbach *et al.*, 2016a; Miles *et al.*, 2007) (Table 2).

Table 2: Most common fungicides used to control *P. pachyrhizi* and their target.

Fungicide	Mode of action	Target gene	Soybean rust resistance
DMI	Sterol biosynthesis impair	sterol 14 $\alpha$ -demethylase of cytochrome P450 ( <i>cyp 51</i> )	Yes (ex: tebuconazol)
Qoi	Inhibition of respiration: complex III	cytochrome b ( <i>cyt b</i> )	Yes (ex: azoxystrobin)
SDHI	Inhibition of respiration: complex II	succinate dehydrogenase ( <i>sdh</i> )	Recently reported

However, the repetitive use of these fungicides (in Brazil, at least 3 fungicide applications per season are necessary) and the adaptability of the pathogen to fungicides have resulted in a decrease in treatment efficacy (Godoy *et al.*, 2016). In Brazil, six years after the first report on the pathogen,

weaker efficacy of DMI was observed, and recently, decreased QoI efficacy has also been reported (Figure 12).

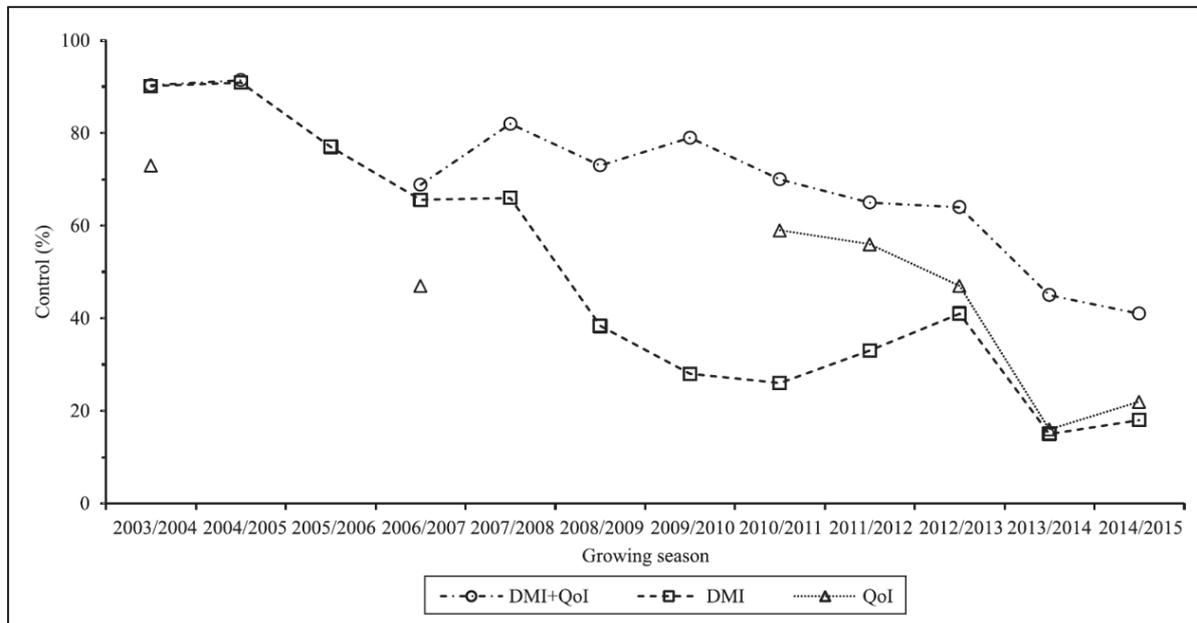


Figure 12: **Decrease in fungicide efficacy in Brazil.** Percentage of Asian soybean rust control in uniform field trials in different soybean-producing regions in Brazil. DMI + QoI, cyproconazole + azoxystrobin; QoI, azoxystrobin; and DMI, tebuconazole. From Godoy *et al.*, 2016.

Resistance to QoI fungicides is mediated by mutations in the cytochrome b-encoding gene (*cyt b*) (Kim *et al.*, 2003). At least three amino acid substitutions are responsible for QoI resistance. These substitutions are as follows: G143A (a glycine replacing an alanine in position 143), F129L (an amino acid change from phenylalanine in position 129) and G137A (an arginine replacing a glycine in position 137) (Schmitz *et al.*, 2014) (Figure 13-a). Although the G143A mutation is the most communally observed in many plant-pathogenic fungi, it is not detectable in rusts, presumably because they possess a type I intron following codon 143. Indeed, a nucleotide substitution in this codon would prevent splicing of the intron, being lethal for those fungi. In contrast, mutation F129L was reported in several plant pathogens, including *P. pachyrhizi*, as a pathway of adaptation for QoI resistance (Klosowski *et al.*, 2016).

The mechanisms of fungal insensitivity to DMIs are variable and complex. This insensitivity can be caused by one or more mutations in the fungal *cyp51* gene (sterol 14 $\alpha$ -demethylase of cytochrome P450), by the overexpression of this gene or by the upregulation of efflux transporters (Schmitz *et al.*, 2014). *Cyp51* gene analyses of Asian soybean rust strains collected in Brazil (year 2010) revealed that point mutations (Figure 13-b) and overexpression were associated with a decreased sensitivity to DMIs. Additionally, some *P. pachyrhizi* isolates showed a constitutive upregulation of efflux transporters (Godoy *et al.*, 2016; Schmitz *et al.*, 2014).

The major mechanism involved in SDHI resistance is due to mutations (generally more than one) in the succinate dehydrogenase *sdh* genes (Sierotzki and Scalliet, 2013). SDHI resistance has been recently reported for *P. pachyrhizi* (Bayer personal communication), probably due to an increasing use of SDHI.

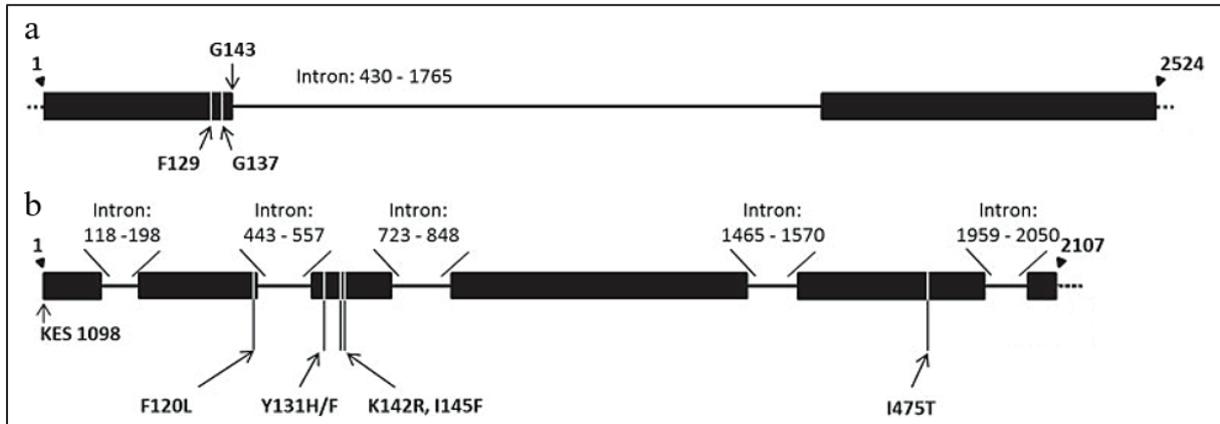


Figure 13: **Schematic gene structure of *P. pachyrhizi* *cyt b* and *cyp51*.** a) *Cyt b* DNA (2524 bp) with an intron following codon 143. The locations of the most important mutations (F129L, G137R and G143A) found in pathogenic fungi are indicated by the arrows. b) *Cyp51* DNA (2107 bp). The mutations found in several *P. pachyrhizi* strains (F120L, Y131H/F, K142R, I145F and I475T) are indicated by arrows. Adapted from Schmitz *et al.*, 2014.

The control of soybean rust with fungicides is only effective with the appropriate application timing. The critical stage to achieve yield potential and therefore for fungicide treatment is from the beginning of flowering of the plant (R1 stage) until the pods are filled (stage R6) (AgBio Communication Unit, 2005) (Figure 14). However, in certain years or areas, the detection of the presence of Asian soybean rust can be earlier (at the vegetative stages) and the control programme must start earlier as well. This fungicide programme protects soybeans from anticipating senescence and seed quality reduction. After the R6 stage, the plant yield has been achieved, and consequently, the control of Asian soybean rust is no longer necessary. Furthermore, rust detection must occur as early as possible because the fungicides used for disease management are principally effective in the early stage of development of the fungus (from spore germination to the beginning of uredinia formation) (AgBio Communication Unit, 2005) and the disease is better contained.

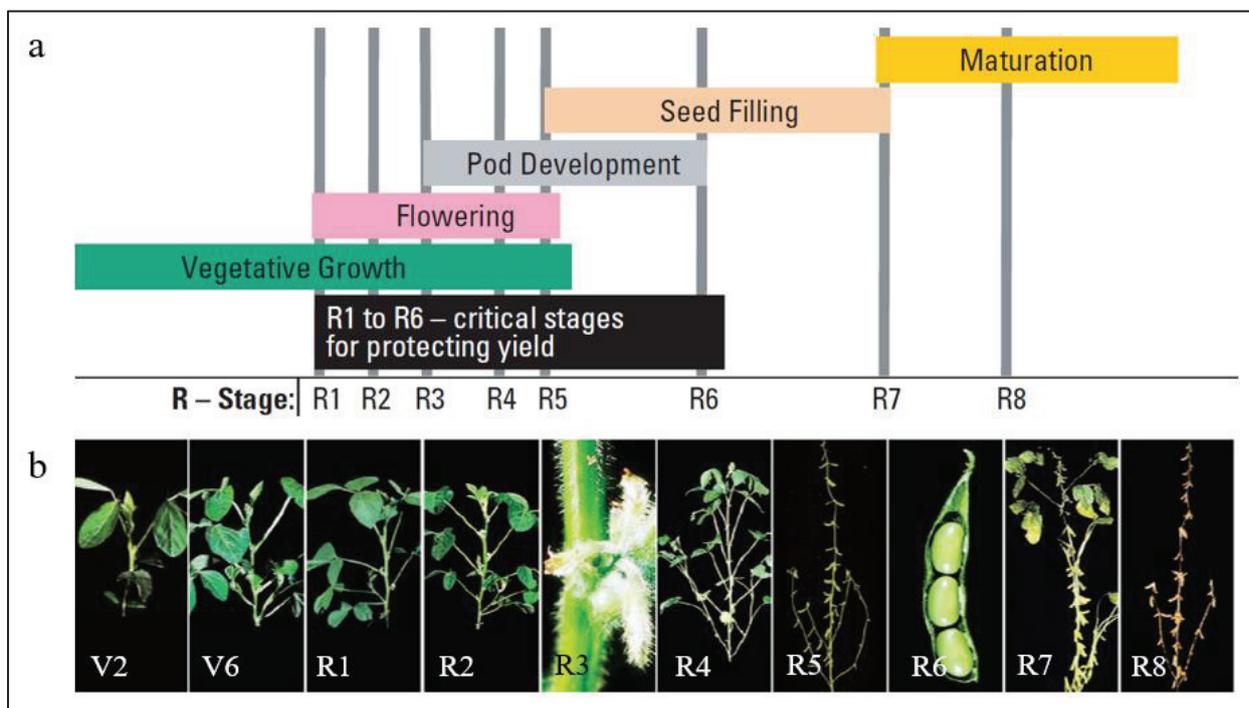


Figure 14: **Soybean development stage.** a) Critical stages for yield protection among the reproductive steps. b) The development stages. V2 and V6: stages with 2 and 6 trifoliates developed, respectively; R1: stage with beginning bloom; full bloom at R2, beginning pods at R3, full pods at R4, beginning of seed formation at R5, full seeds developed at R6, beginning of maturity at R7 and full maturity at R8. From AgBio Communication Unit, 2005.

### Cultural practices

In North America, one technique to determine the presence of the pathogen before it reaches the fields is the establishment of strategically planted soybean pots called “sentinel pots”. Collection and observation of sentinel plots leaves are performed throughout the soybean growing season at regular intervals to define the presence or absence of the disease in different areas. This technique has been used since the beginning of soybean rust reporting and has helped growers as an early warning system for *P. pachyrhizi* infection. The location of the disease is also reported on a public website providing information and modelling on the spread and development of Asian soybean rust (Sikora *et al.*, 2014).

In South America, where the pathogen is present and damages crops every year, periods without soybean plants in the fields (called “vazio sanitario”) have been mandatory in order to reduce the soybean rust inoculum during the off-season. In Brazil, the soybean-free period is from 60 to 90 days between June and November, and in 2016, 21 states adopted this rule (Godoy *et al.*, 2016). Moreover, the remaining plants from the last crop season must be destroyed to reduce the early fungal attack. If this rule is not applied, the grower risks a fine. In Brazil, a second harvest called “safrinha” is possible during the year because of the tropical climate. With same intention as the “vazio sanitario”, a “safrinha” season with soybean has been prohibited, and some states only

allow planting of soybean fields from Sep. 15<sup>th</sup> to Dec. 31<sup>st</sup>. Websites also report the pathogen location, warning farmers about the disease progression. Finally, the control of soybean rust includes the control of alternative hosts of *P. pachyrhizi* such as kudzu plants (Fanaro and Villavicencio, 2011).

### **Breeding**

Other strategies to control the Asian soybean rust disease began to be explored as soon as the disease appeared in the Americas (2001). For this purpose, sources of soybean rust resistance in soybean have been studied with the objective of breeding new varieties. To identify sources of resistance, different germplasm collections of soybean accessions were evaluated against soybean rust isolates. To date, seven dominant *R* genes, named *Rpp1* to *Rpp7* (*Rpp* for Resistance to *P. pachyrhizi*), have been identified (Table 3). The *Rpp1* locus has been reported as conferring an immune response (IM) to isolates of *P. pachyrhizi* from the USA (Hartwig and Bromfield, 1983). *Rpp2* has been identified as allowing red brown (RB) lesions, providing resistance against different rust isolates from Brazil, Australia, India, Taiwan and Philippines (Yu *et al.*, 2015). *Rpp3* has shown to mediate resistance (RB lesion-type) towards different *P. pachyrhizi* isolates (Childs *et al.*, 2018a). The *Rpp4* locus also exhibited RB lesion-type on almost all *P. pachyrhizi* isolates tested. However, heavy sporulation occurred on those lesions, resulting in little field resistance (Walker *et al.*, 2014). Nevertheless, a candidate resistance gene, *Rpp4C4*, coding for a receptor with a nucleotide binding site and a leucine-rich repeat domain (NBS-LRR) has been identified as conferring the resistance to the *Rpp4* locus in accession PI 459025B (Meyer *et al.*, 2009). The *Rpp5* locus showed an RB response to rust isolates from Brazil (Walker *et al.*, 2014), and *Rpp6* has shown good resistance to soybean rust in the USA, providing IM or RB reactions (Miles *et al.*, 2011). Finally, *Rpp7* has been recently found and associated with RB reaction following soybean rust contamination in the USA, Colombia, Paraguay and Australia (Childs *et al.*, 2018b). Ongoing research on these genes allowed the identification of different soybean accessions with these *Rpp* loci (Garcia *et al.*, 2008, 2011; King *et al.*, 2016; Walker *et al.*, 2014; Yamanaka *et al.*, 2016) (Table 3). As noted above, these resistance genes are only effective against specific isolates of *P. pachyrhizi* (Miles *et al.*, 2011), and the resistance conferred by these *Rpp* genes can be overcome by the rust, making breeding solutions very challenging (Yorinori *et al.*, 2005).

Pyramiding *R* genes into one soybean cultivar appears to be a strategy for developing soybean resistance to multiple isolates of *P. pachyrhizi* (Yamanaka *et al.*, 2013). *R* genes are usually identified in different backgrounds, and their introduction in a commercial variety is often associated with a yield drag due to undesirable traits. As a consequence, this approach requires a

pre-selection for all the alleles of the desired *R* genes, which takes time and can limit the probability of selecting higher yielding progenies (Dangl *et al.*, 2013). *R* gene pyramiding can also be defeated in the field, as observed for barley and wheat with *Blumeria graminis* (McDonald and Linde, 2002).

In Brazil, the TMG Company developed soybean cultivars using proprietary markers to select for the *Rpp5* locus, branding it as “Inox Technology”. Those plants exhibited RB resistance against *P. pachyrhizi* due to the *Rpp5* locus ([www.tmg.agr.br](http://www.tmg.agr.br)).

Quantitative trait loci (QTLs) related to soybean rust horizontal resistance could be an alternative to the low durability of resistance associated with *Rpp* genes (Mundt, 2014). In 2008, Silva *et al.* identified 13 QTLs related to 12 traits. Eight of them have been shown to be directly related to plant resistance and therefore could be used in breeding programmes.

However, no soybean cultivars resistant to the various rust isolates are available today to farmers.

## 6) Conclusion

Chemicals are used to reduce and/or inhibit the propagation and development of soybean rust. However, the pathogen might develop resistance to fungicides, and in Brazil, treatment of 34 million hectares of soybean triggers a strong selection pressure. In parallel, soybean *Rpp* genes have been identified for the development of soybean varieties resistant to the pathogen. However, those genes are only effective against specific rust isolates and can be easily overcome. Most of the worldwide cultivated soybean is genetically modified; thus, we can consider transgenic methods to develop soybean cultivars resistant to *P. pachyrhizi*. To this end, biotechnological approaches could contribute to solutions to control Asian soybean rust.

Table 3: Genomic positions and sources of mapped *Rpp* genes.

Gene locus	Chr	Physical position Glyma.Wm82.a2 <sup>a</sup>	Source	References
<i>Rpp1/Rpp1-b</i>	18	56,182,523–56,797,174	PI 200492	Chakraborty et al. (2009); Garcia et al. (2011); Hossain et al. (2014); Hyten et al. (2007); Kim et al. (2012); McLean and Byth (1980); Ray et al. (2009); Rocha et al. (2015); Yamanaka, Morishita et al. (2015); Yamanaka et al. (2016)
			PI 594538A	
			PI 587886	
			PI 587880A	
			PI 594760B	
			PI 561356	
<i>Rpp2/rpp2</i>	16	27,937,049–30,478,472	PI 594767A	
			PI 587905	
			PI 594177	
			PI 587855	
<i>Rpp3/Rpp?</i> ( <i>Hyyuga</i> )	6	44,049,891–45,995,029	PI 230970	Bromfield and Hartwig (1980); Calvo et al. (2008); Garcia et al. (2008); Hartwig and Bromfield (1983); Nogueira et al. (2008); Silva et al. (2008); Yu et al. (2015)
			PI 462312	
			PI 506764 <sup>b</sup>	
<i>Rpp4/Rpp4-b</i>	18	51,397,064–51,584,617	PI 628932	Brogini (2005); Hartwig and Bromfield (1983); Hossain et al. (2014); Hyten et al. (2009); Kendrick et al. (2011); Monteros et al. (2007); Ray et al. (2011)
			PI 567099A	
			PI 416764	
			PI 423972	
<i>Rpp5/rpp5</i>	3	29,862,641–32,670,690	PI 459025B	Garcia et al. (2008); Hartwig (1986); King et al. (2017); Meyer et al. (2009); Silva et al. (2008)
			PI 200526	Calvo et al. (2008); Garcia et al. (2008); Kendrick et al. (2011)
			PI 200456	
<i>Rpp6/Rpp</i> [PI567068A]	18	5,953,237–6,898,528	PI 200487	
			PI 471904	
			PI 506764 <sup>b</sup>	
<i>Rpp7</i>	19	39,462,291–39,616,643	PI 567102B	King et al. (2015); Li et al. (2012); Liu et al. (2016)
			PI 567068A	
			PI 567104B	
			PI 605823	Childs et al. (2018)

a: Physical positions of the SNP markers used for mapping were taken from <http://www.soybase.org>. b: PI 506764 has *Rpp* resistance alleles at the *Rpp3* and *Rpp5* loci. From Childs *et al.*, 2018.

## D. PLANT DISEASE ENGINEERING

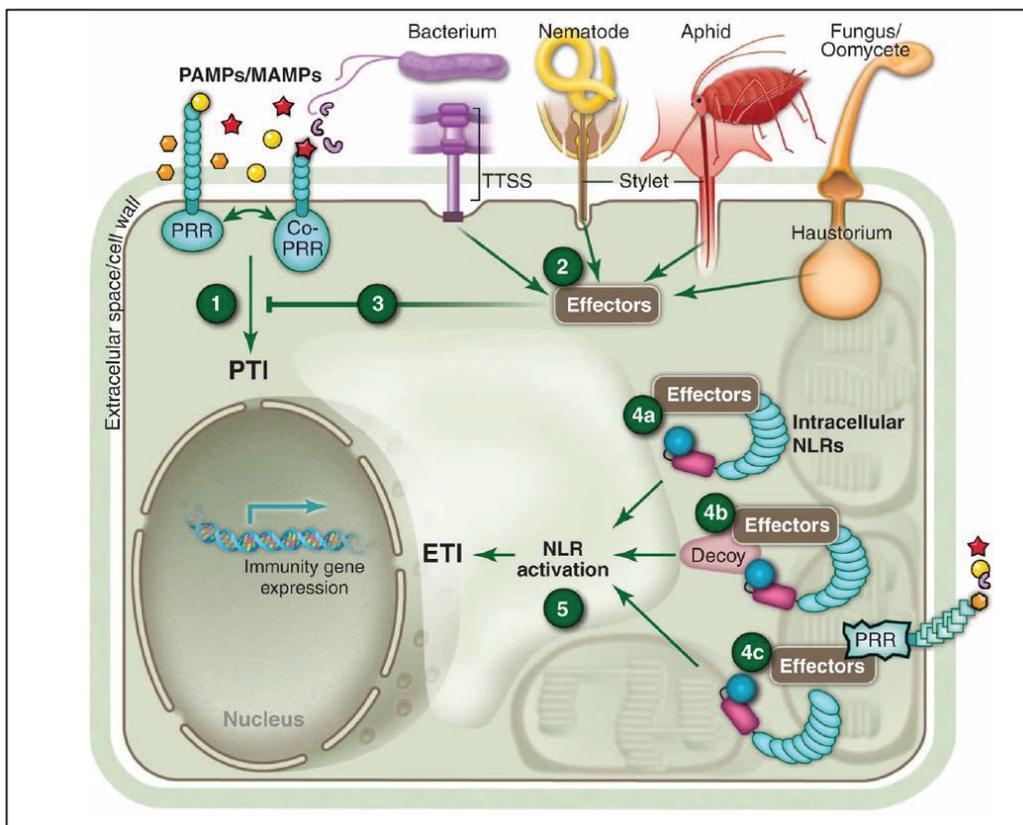
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Genetic engineering has the potential to increase plant resistance to pathogens by inserting one or multiple genes in the plant genome. Mechanisms of plant defence have been largely studied, furthering the development of this approach. The expansion of molecular biology in the early 1980s allowed remarkable advances in elucidating the plant mechanisms involved in the response to pathogen infection (Wally and Punja, 2010). This knowledge served as a basis for transgenic approaches aiming to increase plant defence.

### 1) General plant defence overview

Confronted with microbial infections, plants have evolved multiple complex defence mechanisms. First, pathogen (or microbial) associated molecular patterns (PAMPs or MAMPs) from the pathogen (bacterial flagellin or fungal chitin) (Chisholm *et al.*, 2006) are recognized by the plant through membrane pattern recognition receptors (PRRs). Several PRRs have been well characterized. In *Arabidopsis*, the chitin elicitor receptor kinase (CERK1) perceives chitin oligomers and the flagellin sensing 2 (FLS2) recognizes and binds the 22-amino acid epitope of the bacterial flagellin N-terminus (flg22) (Silva *et al.*, 2018). Damage associated molecular patterns (DAMPs) resulting from plant cell wall degradation by a pathogen can also be detected (Dodds and Rathjen, 2010). PRR stimulation leads to the activation of PAMP-triggered immunity (PTI), the first level of basal plant defence (Figure 15, step 1). This global response involves the recognition of PAMPs/MAMPs that are evolutionarily conserved across a class of organisms and therefore allows resistance to a majority of pathogens (Silva *et al.*, 2018). Nevertheless, some microorganisms are able to overcome this first level of defence. Well-adapted pathogens secrete a plethora of effectors that modulate host cell mechanisms and physiology (Figure 15, step 2) and can suppress PTI (Figure 15, step 3) through susceptibility (S) proteins (effector targets). This suppressed state leads to the infection of the host cell and results in effector-triggered susceptibility (ETS) (Silva *et al.*, 2018). Effectors can be delivered inside the cell apoplast (and block PAMP/MAMP perception) or inside the cell cytoplasm (Dangl *et al.*, 2013). Following the bypass by the pathogen of the PTI, a second step of specific defence, effector-triggered immunity (ETI), can be activated by the detection of specific effectors considered as avirulence (Avr) proteins (Jones and Dangl, 2006) (Figure 15, step 5). Avr detection occurs via intracellular resistance proteins (R proteins). Most of those proteins

are part of the nucleotide-binding leucine-rich repeat (NLR) receptor composed of one nucleotide binding site (NBS) and one leucine-rich repeat (LRR) domain (Cui *et al.*, 2015). Detection of effectors by the R proteins can occur via direct or indirect interaction (Figure 15, step 4a, 4b and 4c) (Dangl *et al.*, 2013). The ETI response is similar to the PTI response but occurs more rapidly and with greater intensity (Cui *et al.*, 2015; Dodds and Rathjen, 2010). ETI is specific to some pathogens that secrete an Avr product and frequently involves localized programmed cell death, known as the hypersensitive response (HR). The role of the HR is to reduce pathogen development inside plant tissues (Dodds and Rathjen, 2010).



**Figure 15: Schematic of the plant immune system.** Pathogens of all lifestyle classes express PAMPs and MAMPs as they colonize plants. Plants perceive these pathogens via extracellular PRRs and initiate PTI (step 1). Pathogens deliver virulence effectors to both the plant cell apoplast and to the plant cell interior (step 2). These effectors are addressed to specific subcellular locations, where they can suppress PTI and facilitate virulence (step 3). Intracellular NLR receptors can sense effectors in three principal ways: first, by direct receptor ligand interaction (step 4a); second, by sensing effector-mediated alteration in a decoy protein that structurally mimics an effector target but has no other function in the plant cell (step 4b); and third, by sensing effector-mediated alteration of a host virulence target, such as the cytosolic domain of a PRR (step 4c). This process results in ETI. From Dangl *et al.*, 2013.

According to the gene-for-gene concept of Flor (Flor, 1971), for one *R* gene of the plant, there is a gene encoding for an Avr protein in the pathogen genome. The interaction between the *R* and Avr proteins triggers host resistance. However, if no interaction occurs, the pathogen can

grow in plant tissues, leading to symptom development. The plant is then susceptible to the pathogen. Some microorganisms have evolved to overcome the ETI and promote ETS by losing or modifying their Avr protein or by creating new undetected effectors. The host is then subjected to a new selection pressure, leading to the appearance of new *R* genes to promote ETI again (Jones and Dangl, 2006). The “zig-zag” model describes this coevolution between plant and pathogen (Figure 16).

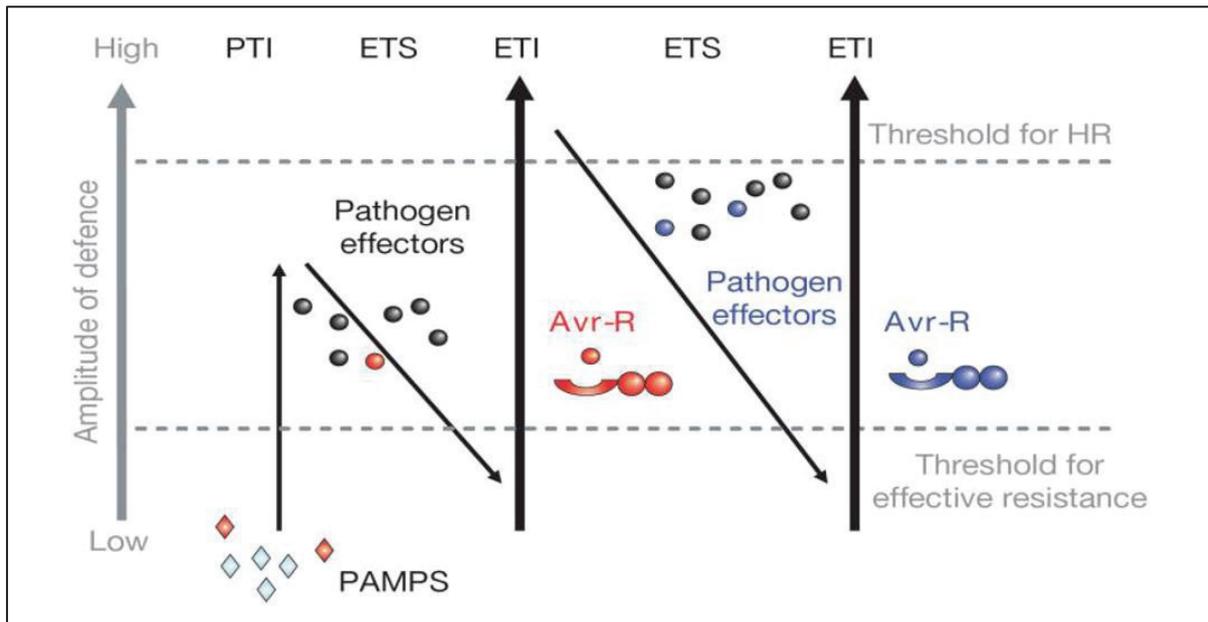


Figure 16: “Zig-zag” model. First, detection of pathogen associated molecular patterns via the PRRs proteins leads to the PTI. Then, adapted pathogens can deliver effectors that contribute to ETS for effector-triggered sensibility. When these effectors are recognized by NB-LRR proteins, the ETI is activated. Pathogens have evolved to lose the recognized effector (in red) and acquire new effectors (in blue) that will promote ETI suppression. Selection favours the emergence of new NB-LRR plant alleles to recognize new effectors and promote ETI again. From Jones and Dangl 2006.

Furthermore, pathogen recognition in plants can increase the production of reactive oxygen species (ROS) and nitric oxide (NO), triggered by calcium ( $\text{Ca}^{2+}$ ). An early increase in cytosolic  $\text{Ca}^{2+}$  concentration is induced by several extracellular stimuli, such as exogenous  $\text{H}_2\text{O}_2$ , PAMPs/MAMPs, effectors or DAMPs. These changes are detected by  $\text{Ca}^{2+}$  sensors (such as calmodulin or calmodulin-like protein and calcium-dependent protein kinase) and transduced into a signal, which leads to the HR, gene regulation related to stress responses, as well as rapid production of  $\text{H}_2\text{O}_2$  and NO. Thus,  $\text{Ca}^{2+}$  signalling is intrinsically linked with early PTI/ETI perception mechanisms (Silva *et al.*, 2018).

In addition, both plants and pathogens produce small non-coding RNAs (sRNAs) involved in plant-pathogen interactions; among them, miRNAs are common regulators of endogenous pathways that can be involved in defence and/or pathogenicity and siRNAs are often produced

to suppress exogenous RNA, resulting from transposons, overexpressed transcripts or virus infection. This effect is realized through the RNA silencing mechanisms, also called RNA interference (RNAi) (Weiberg *et al.*, 2014). RNA silencing suppressors have been identified in plant pathogens, suggesting that disruption of host silencing is a strategy to promote disease. As an evolutionary counteraction, plants developed specific defence against RNA-silencing suppression by pathogens, providing another illustration of the molecular arms race between plants and pathogens (Pumplin and Voinnet, 2013).

Moreover, fungal infection can induce different plant hormone pathways depending on the lifestyle of the pathogen. Plant pathogens can be divided into biotrophs that feed on living host tissue and necrotrophs that derive nutrients from dead or dying host cells (Glazebrook, 2005). Usually, salicylate signalling is implicated in defence against biotrophic fungi, and jasmonate together with ethylene participate in the defence against necrotrophic fungi (Robert-Seilaniantz *et al.*, 2007). Other phytohormones, such as abscisic acid (ABA), auxin, gibberellins (GAs), cytokinins (CKs), and brassinosteroids (BRs), are also involved in defence responses (Berens *et al.*, 2017).

## 2) Soybean reactions to *P. pachyrhizi* infection

According to the described plant immunity mechanism, reactions of the soybean host plant to *P. pachyrhizi* have been classified in 3 types. The first type is the immune (IM) resistance without any visible disease symptoms. In kudzu, this immune response is associated with the HR limited to one/few cells of the epidermis and coupled with encasement of the penetrated hypha with cell wall deposition (CWD). According to Jordan *et al.*, (2010) the primary component of the CDW is likely callose (Figure 17-a). The second reaction type is resistance, which leads to the development of red brown (RB) lesions. RB lesions are the result of a multicellular HR that affects epidermal and mesophyll cells, limiting colonization and spore production (Figure 17-b). Both of those reactions are called incompatible because fungal growth inside host tissues is blocked. According to the gene-for-gene theory, IM and RB reaction are initiated by the perception of a fungal Avr protein by an R gene of the host plant (Echeveste da Rosa, 2015). Susceptibility is characterized by (TAN) lesions with host tissues colonized by the pathogen (Figure 17-c). This type of reaction is called compatible. It has been reported that RB lesions have a longer latency period, are smaller and have fewer uredinia than the TAN lesions (Echeveste da Rosa, 2015). A gradient in disease severity and sporulation level exists between RB and TAN reactions (Miles *et al.*, 2011).

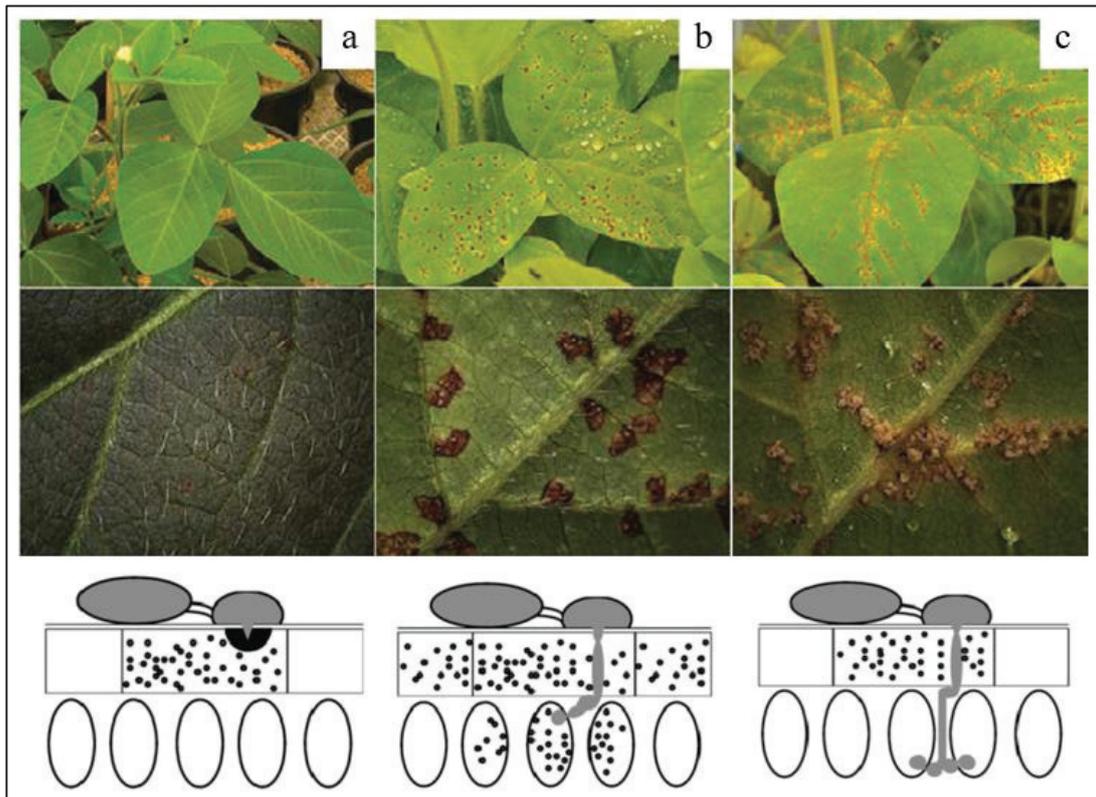


Figure 17: **Soybean response to *P. pachyrhizi* infection.** a) Immunity, b), partial resistance with RB lesions and c) susceptibility and TAN lesions. Dead cells are shown with small brown dots. The drawings represent what is observed in Kudzu. Adapted from Echeveste da Rosa, 2015, and Jordan *et al.*, 2010.

To make the link between phenotypic observations and molecular mechanisms, gene expression during rust infection in soybean has been investigated. It has been reported that *P. pachyrhizi* contamination induces a biphasic response in soybean (van de Mortel *et al.*, 2007; Schneider *et al.*, 2011) (Figure 18). A first burst of soybean gene deregulation in compatible and incompatible interactions occurs at the beginning of the infection (6/12 hpi). This first burst in gene expression is correlated with fungal germination, appressorium formation and penetration in the epidermal cells (Schneider *et al.*, 2011). This early response to the rust suggests a non-specific recognition of the pathogen and the activation of basal plant defence similar to PTI. This non-specific recognition may be activated by PAMPs and/or DAMPs (Dodds and Rathjen, 2010; van de Mortel *et al.*, 2007). However, after this period, the soybean rust is still able to grow in plants where an incompatible interaction occurs. These observations suggest that the pathogen can suppress or evade this first defence response, remaining undetectable after its penetration into the leaf mesophyll (Schneider *et al.*, 2011). Then, a second phase of gene expression occurs after haustorium formation. In susceptible plants (compatible interaction), this second burst of gene deregulation occurs at 96 hpi, while in resistant plants (incompatible interaction), it is observed earlier, at 72 hpi. Van de Mortel *et al.* suggested that the early

activation of this second gene deregulation prevents the rapid growth of the fungus as it is correlated with the inhibition of haustorium proliferation. This biphasic gene expression has been observed in the interaction of three different soybean genotypes with three different *P. pachyrhizi* isolates (Schneider *et al.*, 2011).

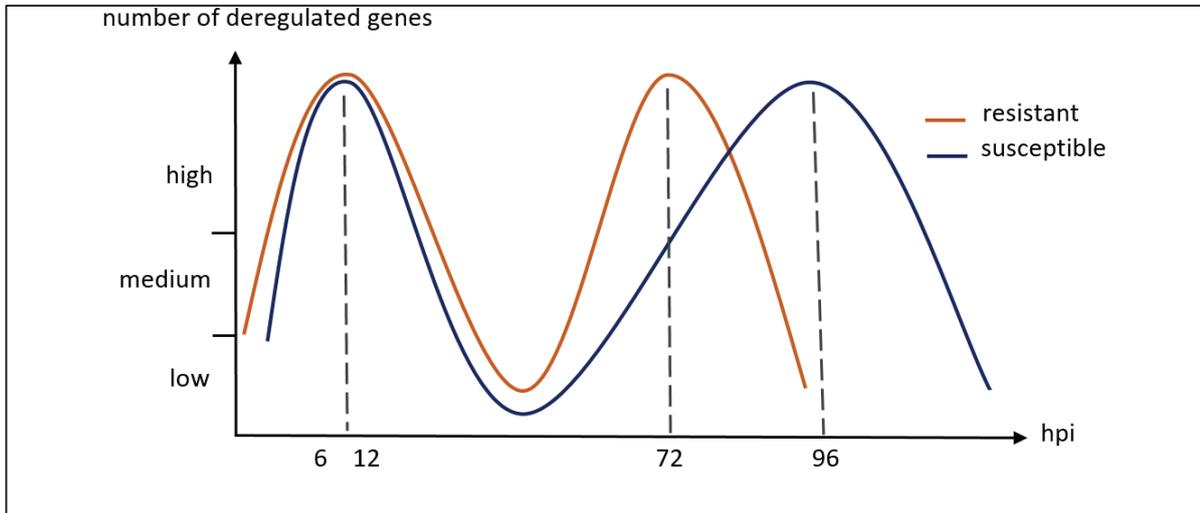


Figure 18: **Soybean biphasic response to *P. pachyrhizi*.** A first burst of deregulation is observed at 6/12 hpi and a second one at 72 hpi in susceptible plants and at 92 hpi in resistant plants. According to Schneider *et al.*, 2006.

The majority (75%) of the deregulated genes identified in the first and second responses were upregulated during both compatible and incompatible interactions (Schneider *et al.*, 2011). According to van de Mortel *et al.*, signal pathways associated with plant defence are activated upon pathogen recognition in both interactions. This process includes changes in  $\text{Ca}^{2+}$  levels, production of ROS and activation of MAPKs. In addition, defence response genes such as PR or lipases were over-represented in deregulated genes (Schneider *et al.*, 2011). An induction of the phenylpropanoid and flavonoid pathway is observed following the biphasic response under soybean rust infection. This pathway is involved in the biosynthesis of phytoalexins and antimicrobial compounds (van de Mortel *et al.*, 2007). Another category of genes is the transcription factors (TFs), with WKY and NAC TFs being nearly all upregulated in the two interactions. The AUX-IAA-ARF and MYB-HD classes of transcription factors appear to be exclusively downregulated in both compatible and incompatible interactions (Schneider *et al.*, 2011). AUX-IAA-ARF and MYB-HD transcription factors are involved in the regulation of plant growth and development, and their downregulation is consistent with the idea that those processes are negatively affected during pathogen defence (Schneider *et al.*, 2011). The increased expression of WKY and NAC TFs indicates that they are needed to control gene response (Schneider *et al.*, 2011). Panthee *et al.* (2007) reported the induction of two SA-related

genes at 72 hpi. Genes implicated in photosynthesis and various transport processes (such as ammonium and  $\text{Ca}^{2+}$  transport) were also deregulated following *P. pachyrhizi* infection (Schneider *et al.*, 2011).

In addition, other studies have focused on the susceptible soybean gene expression 10 days after inoculation with *P. pachyrhizi*. In those studies, genes related to the defence response were also upregulated, suggesting that the plant is still fighting against the pathogen even at the end of the infection process. Tremblay *et al.* (2010) reported the activation of SA- and JA-responsive genes and genes involved in JA biosynthesis at 10 dpi in susceptible plants. Many genes involved in metabolic pathways, including carbohydrate, amino acid and lipid metabolism, have been shown to be downregulated. This demonstrates that soybean rust strongly affects plant metabolism at a later stage of infection (Tremblay *et al.*, 2010, 2011).

These transcriptomic studies provide a better understanding of the interaction between the pathogen and the plant and highlight the soybean genes implicated in the defence response suitable for the development of biotechnological approaches.

### **3) Biotechnological approaches to control *P. pachyrhizi***

Transgenic model plants and crops expressing plant defence genes have been produced and evaluated for their ability to confer disease resistance (Gurr and Rushton, 2005a). For instance, expression in tomato (*Solanum lycopersicum*) of an *R* gene (*Bs2*) from pepper provides resistance to multiple *Xanthomonas* species (Horvath *et al.*, 2012). Expression in *Nicotiana benthamiana* and *Solanum lycopersicum* of a PRR gene (*ERF*) from *Arabidopsis* conferred resistance to different bacteria (*Ralstonia solanacearum* and *Xanthomonas perforans*) (Lacombe *et al.*, 2010). The study of these genes resulted in the development of different transgenic approaches suitable for plant disease engineering (Gurr and Rushton, 2005a; Islam, 2006) and potentially for *P. pachyrhizi* control.

A first strategy involves targeting the fungus via the expression of RNA interference (1) or antifungal peptides (2). The second strategy focuses on plant defence modulation by modulating structural defence (3) R (4) or NHR (5) genes, signal regulation (6), defense pathways (7) or engineering cell death (8).

#### **(1) RNA interference (RNAi)**

The silencing of essential gene(s) of a pathogen via RNAi has been developed to improve host protection (Rosa *et al.*, 2018). This approach, called host-induced gene silencing (HIGS), has

been shown to be successful against fungal plant pathogens (Rosa *et al.*, 2018). In wheat, stable transgenic plants carrying an RNAi construct against the  $\beta$  1-3-glucan synthase gene (*FcGls1*) of *Fusarium culmorum* showed enhanced resistance to the pathogen following leaf and spike inoculation (Chen *et al.*, 2016). Similar results were obtained with transgenic plants carrying a triple combination of *FcGls1*-, *FcChsV* (a chitin synthase)- and *FcFmk1* (a MAPK)-targeted genes (Chen *et al.*, 2016). Also in wheat, rust disease caused by *Puccinia triticina* has been strongly and durably reduced by silencing a MAPK kinase gene (*PsFUZ7*) (Zhu *et al.*, 2017). In *Uromyces fabae*, the *HXT1* gene (a hexose transporter) has been shown to be almost preferably expressed in rust haustoria, and it was suggested that this gene is essential for the development of the rust fungus on its host (Voegelé *et al.*, 2001). In the WO 2017/016963 A1 patent (Gautier *et al.*, 2017), the *HXT1*-homologue of *P. pachyrhizi* has been silenced in soybean by using the VIGS (virus-induced gene silencing) technology. This silencing resulted in a decrease in the expression of the corresponding mRNA. Effector proteins implicated in *P. pachyrhizi* pathogenicity and targeting soybean immunity could also be considered as promising targets to be silenced. *P. pachyrhizi* effector candidates (*PpECs*) have been identified through transcriptomic analysis using isolated pathogen haustoria (Link *et al.*, 2014). One of them (*PpEC23*) was shown to suppress the HR and basal defence response. *PpEC23* was reported as interacting with the soybean transcription factor *GmSPL121* that negatively regulates soybean defence response (Qi *et al.*, 2016). In addition, 17 other *PpECs* were identified as suppressors of PTI (Qi *et al.*, 2017).

## **(2) Expression of antimicrobial compounds**

A commonly used approach for engineering fungal resistance is the expression of pathogenesis-related (PR) proteins and antimicrobial peptides. PR proteins are produced and accumulated in notable quantities during pathogen attacks, and they are classified into 17 groups from PR-1 to 17 (Ebrahim *et al.*, 2011). Chitinases (PR-3, 4, 8 and 11) and glucanase (PR-2) represent a subset of pathogenesis-related proteins. These two types of proteins have the ability to hydrolyse chitin, a major component of the fungal cell wall (Grover, 2012). Many plant chitinases and glucanases have been reported to possess antifungal activity. Chitinases have been largely studied for the development of transgenic plants (Kumar *et al.*, 2003). Fewer reports of transgenic plants with glucanases are available; however, combined expression of chitinases and glucanases genes showed a higher level of resistance than expression of either gene alone (Ceasar and Ignacimuthu, 2012). Defensins (PR-12) are small cysteine-rich peptides also used for their antifungal activity. A recombinant pea defensin, rDrr230a, has been shown to be active against pathogenic fungi, including *P. pachyrhizi*. This defensin is able to inhibit

100% of *in vitro* germination of soybean rust spores (Figure 19-a) (Lacerda *et al.*, 2016). In addition, plant phytoalexins, known as antimicrobial compounds, are produced following pathogen attacks. A phytoalexin (medicarpin) has been reported to be able to inhibit the spore germination and appressorium formation of *P. pachyrhizi* (Figure 19-b) (Ishiga *et al.*, 2015). The phenylpropanoid pathway plays an important role in phytoalexin production. It has been observed that the silencing of a gene implicated in this pathway (*GmPAL1*) in *Rpp2* plants led to a susceptible phenotype, revealing that *GmPAL1* is implicated in *Rpp2*-mediated disease resistance against *P. pachyrhizi* (Pandey *et al.*, 2011). The expression of genes involved in the phenylpropanoid pathway was also reported to be induced in soybean carrying the *Rpp3* locus (Schneider *et al.*, 2011), showing that overproduction of phytoalexins could be a strategy for plant engineering.

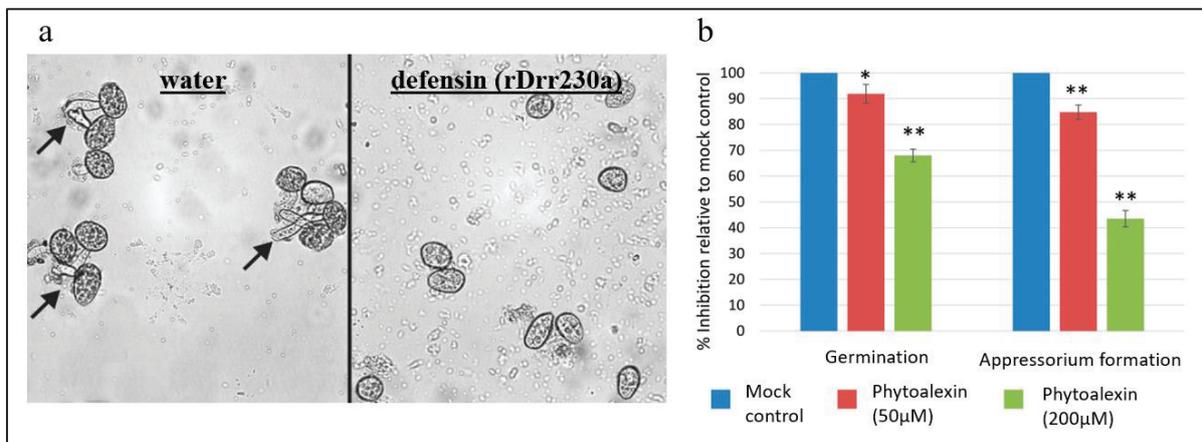


Figure 19: **Impact of antimicrobial peptide on *P. pachyrhizi*** a) Inhibition of spore germination of *P. pachyrhizi* by rDrr230a defensin. Arrows represent *P. pachyrhizi* germinating spores. Adapted from Lacerda *et al.*, 2016. b) A phytoalexin (medicarpin) inhibits soybean rust pre-infection structures. Percentage of germination and appressorium formation (means  $\pm$  standard deviation) 18 h after treatments. Asterisks indicate a significant difference (paired Student's *t* test, \**P* < 0.05, \*\**P* < 0.01). Adapted from Ishiga *et al.*, 2015.

### **(3) Increasing structural defence**

The cell wall not only provides structure to the plant but also plays an important role in preventing pathogen invasion of the plant. Lignin is part of the cell wall, and its deposition in infected cells may prevent the spread of pathogenic toxins and enzymes (Miedes *et al.*, 2014). In cotton, overexpression of a gene enhancing lignification (*Dirigent1*) blocked the spread of the wilt fungus *Verticillium dahlia* (Shi *et al.*, 2012). Additionally, the phenylpropanoid pathway plays an important role in lignin biosynthesis. It was reported that *Rpp2* soybean plants in which a gene implicated in lignin biosynthesis (*GmO-MT*) was silenced had a susceptible phenotype and a reduced lignin content (Pandey *et al.*, 2011).

#### **(4) Expression of resistance genes**

Plant breeding improvement for disease control is mainly based on the use of *R* genes. The advantage of genetic engineering approaches is the possibility to introduce any *R* gene from unrelated plant species. This solution seems relevant for the control of *P. pachyrhizi* due to the limited *Rpp* genes in soybean germplasm and the rapid breakdown of these genes by the pathogen. Kawashima *et al.* (2016) identified in *Cajanus cajan* an *R* gene (*CcRpp1*) encoding an intracellular immune receptor and conferring resistance to *P. pachyrhizi*. Heterozygous and homozygous transgenic soybean plants expressing this gene showed a reduction of lesions and an immunity reaction to *P. pachyrhizi* isolates from North America, respectively (Figure 20a-b). This observation was correlated with the relative expression of *CcRpp1*, which was highest in the homozygous plants (Figure 20-c). Furthermore, preliminary data indicated that the plant fitness of transgenic soybean expressing *CcRpp1* was not impacted (Figure 20-d).

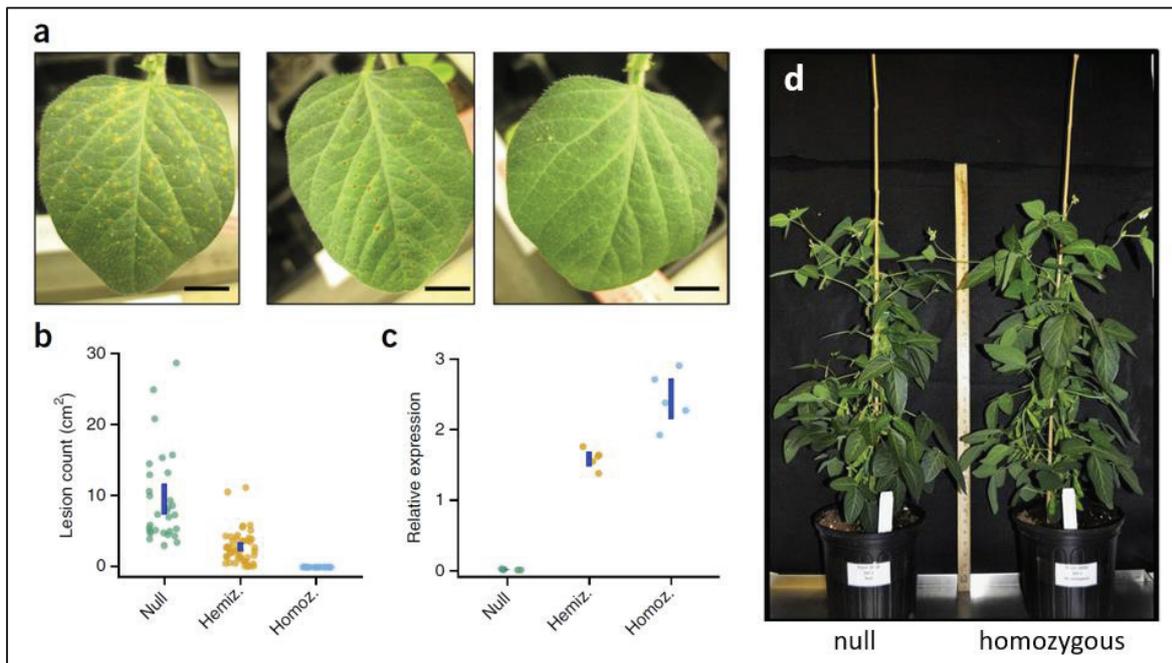


Figure 20: A *CcRpp1* gene from *C. cajan* conferring resistance to the soybean rust a) Soybean plants that express the *CcRpp1* gene (driven by the *G. max* SUBI-1 promoter) challenged with *P. pachyrhizi*. From left to right, T<sub>1</sub> null plants, T<sub>1</sub> hemizygous plants and T<sub>1</sub> plants homozygous for the transgene. Scale bars, 1 cm. b) Average lesion count per cm<sup>2</sup> of leaves monitored in T<sub>1</sub> null plants, T<sub>1</sub> hemizygous plants and T<sub>1</sub> plants homozygous for the transgene. c) Expression level differences between T<sub>1</sub> null plants, T<sub>1</sub> hemizygous plants and T<sub>1</sub> plants homozygous for the *CcRpp1* gene as determined by qRT-PCR. d) Null segregants compared to plants expressing *CcRpp1* at 8 weeks after germination. Adapted from Kawashima *et al.*, 2016.

#### **(5) Expression of non-host resistance genes**

Non-host resistance (NHR) is defined as the resistance of all genotypes of a non-host plant to all genotypes/races of fungal species. Since this type of resistance is broad spectrum and

urable, NHR has a high value for crop improvement. Studies have revealed that multiple genes are implicated in this resistance type (Lee *et al.*, 2016), and the potential of NHR genes to increase resistance has been shown in many crops using genetic engineering (Gill *et al.*, 2015). NHR against *P. pachyrhizi* has been well studied, particularly in *Arabidopsis thaliana* (Goellner *et al.*, 2010). Loehrer *et al.* revealed that the pathogen activates marker genes of necrotrophic infection such as the *PDF1.2* defensin (Loehrer *et al.*, 2008). It has been suggested that the fungus would mimic a necrotrophic behaviour to promote its development inside the host tissues (Campe *et al.*, 2014). *A. thaliana* wild-type plants infected by the soybean rust showed no symptoms. On those plants, *P. pachyrhizi* was able to form an appressorium and penetrate inside the epidermal cells, but the fungal development stopped at the mesophyll border (Figure 21: category I). It has been shown that epidermal penetration resistance of *A. thaliana* to *Phakopsora pachyrhizi* requires *PEN1*, *PEN2* and *PEN3* genes (*PEN* for *PENETRATION*). Indeed, in *pen2* mutants, *P. pachyrhizi* overcomes *Arabidopsis* pre-invasion NHR and colonizes the mesophyll. Nevertheless, the development of haustoria was only rarely observed (in 5-10% of the interaction sites) in *pen2* plants infected by the pathogen (Figure 21: category II). This finding indicated that post-invasion mesophyll defence is intact in this mutant. Furthermore, post-invasion resistance in the mesophyll depends on the combined functionality of *PEN2*, *PAD4* (phytoalexin-deficient 4) and *SAG101* (senescence-associated gene 101). As a consequence, in the triple-mutant *pen2 pad4 sag101*, the soybean rust colonized the mesophyll and haustoria in 30-50% of the interaction sites (Figure 21: category III) (Langenbach *et al.*, 2013).

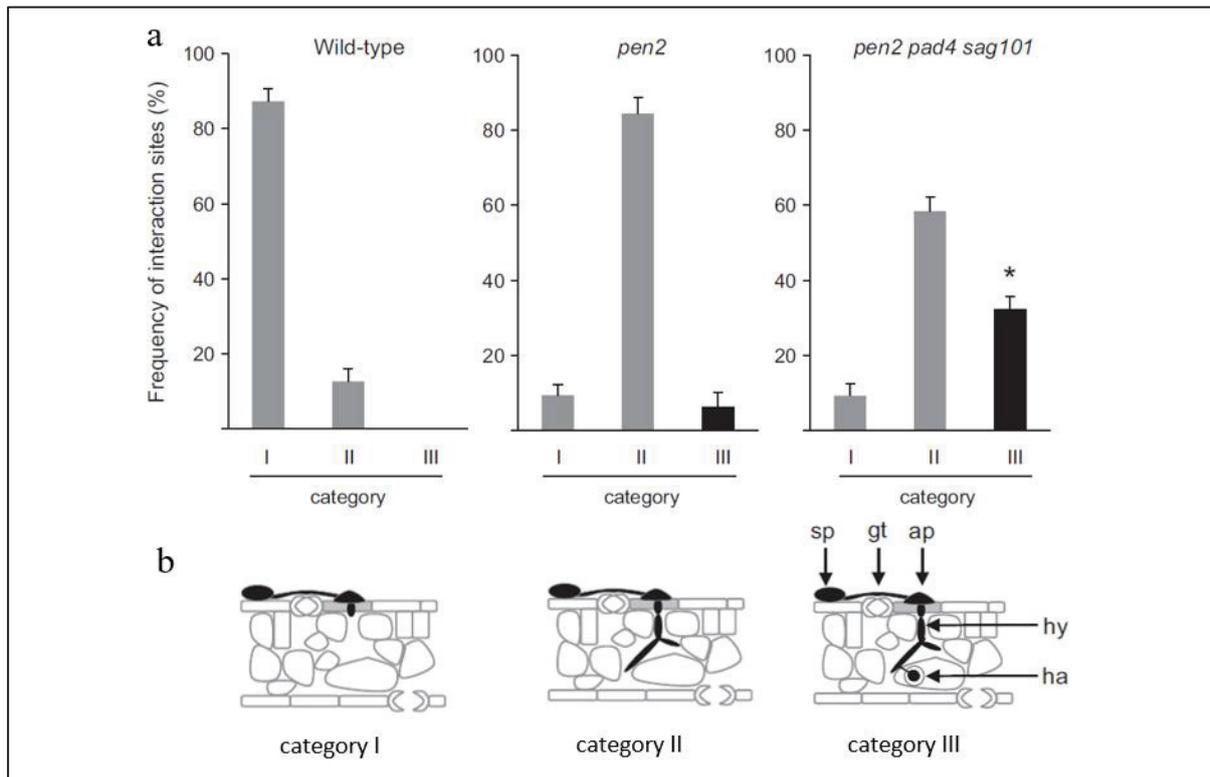


Figure 21: *pen2* and *pen2 pad4 sag101* mutants of *A. thaliana* are impaired in pre- or pre- and post-invasion NHR to *P. pachyrhizi*. a) Fungal development was analysed and assigned to one of three categories (I–III). Average values are shown. Error bars indicate standard deviation (n = 900). The asterisk represents a significant difference in haustorium abundance ( $P < 0.001$ ) between *pen2* and *pen2 pad4 sag101* according to Student's t test. b) Schematic overview. Black: fungal structures; grey: dying epidermal cell. ap, appressorium; gt, germ tube; ha, haustorium; hy, hypha; sp, spore. Adapted from Langenbach *et al.*, 2013.

Different genes with a role in *A. thaliana* post-invasion mesophyll resistance to *P. pachyrhizi* have been identified (Langenbach *et al.*, 2016b; Schultheiss *et al.*, 2013), including in the *A. thaliana* *CL4* gene (putative leucine-rich repeat protein kinase). *CL4* inhibition in *Arabidopsis pen2* mutants led to higher haustoria formation (30–40%) after infection with *P. pachyrhizi*. BASF company showed that the overexpression of this gene in soybean resulted in higher resistance to *P. pachyrhizi* (patent WO/2013/09738 A1, Schultheiss *et al.*, 2013). In T1 transgenic lines overexpressing *CL4*, 10% reduction of the disease leaf area compared to wild-type plants has been observed.

## **(6) Signal regulation**

The modification of existing signalling pathways has the advantage of activating a large number of genes compared to the overexpression of a single gene or a small group of genes. Transcription factors appear as good candidates for this purpose as they control the expression of several genes in a single pathway (Yanagisawa, 1998). For example, the WRKY transcription

factors have been recognized for their role in increasing disease resistance. This effect is illustrated by the overexpression of *A. thaliana* *WRKY70* that provides enhanced resistance to powdery mildew (Li *et al.*, 2006). Furthermore, in the *Rpp2* soybean background, silencing of three genes coding for WRKY transcription factors (*GmWRKY36*, *GmWRKY40* and *GmWRKY45*) enhanced the susceptibility to *P. pachyrhizi* (Figure 22) (Pandey *et al.*, 2011).

### **(7) Modulation of the defence pathways**

Signalling molecules implicated in hormone defence pathways have been recognized for their ability to promote plant resistance. For example, NPR1, EDS1 and PAD4, controlling the salicylic acid defence pathway, have been identified as controlling *Rpp2*-mediated resistance in soybean (Figure 22) (Pandey *et al.*, 2011). Virus-induced silencing of those genes in an *Rpp2* soybean cultivar led to the development of TAN lesions. PAD4 was also required for *A. thaliana* NHR to the soybean rust (Langenbach *et al.*, 2013). Therefore, SA accumulation seemed to be effective for the growth inhibition of *P. pachyrhizi* in soybean according to Pandey *et al.* (2011).

In pepper (*Capsicum annuum*), the *CASAR82A* gene was required for the induction of systemic acquired resistance (SAR), a long-distance signal that provides immunity in non-infected tissues against a broad range of pathogens (Lee and Hwang, 2005). In *A. thaliana*, the overexpression of *CASAR82A* was accompanied by ectopic expression of PR genes, faster growth and resistance to different pathogens (*Pseudomonas syringae* pv. *tomato*, *Fusarium oxysporum* f.sp. *matthiolae* and *Botrytis cinerea*) (Lee and Hwang, 2006). The BASF company has developed transgenic soybeans expressing *CASAR82A* under the control of a constitutive promoter (patent US 2014/0137283 A1, Schultheiss *et al.*, 2018). The overexpression of this protein increased soybean resistance against *P. pachyrhizi* (Schultheiss *et al.*, 2018). In transgenic soybeans, the leaf area covered by the disease was 44.5% reduced compared to that in the wild type.

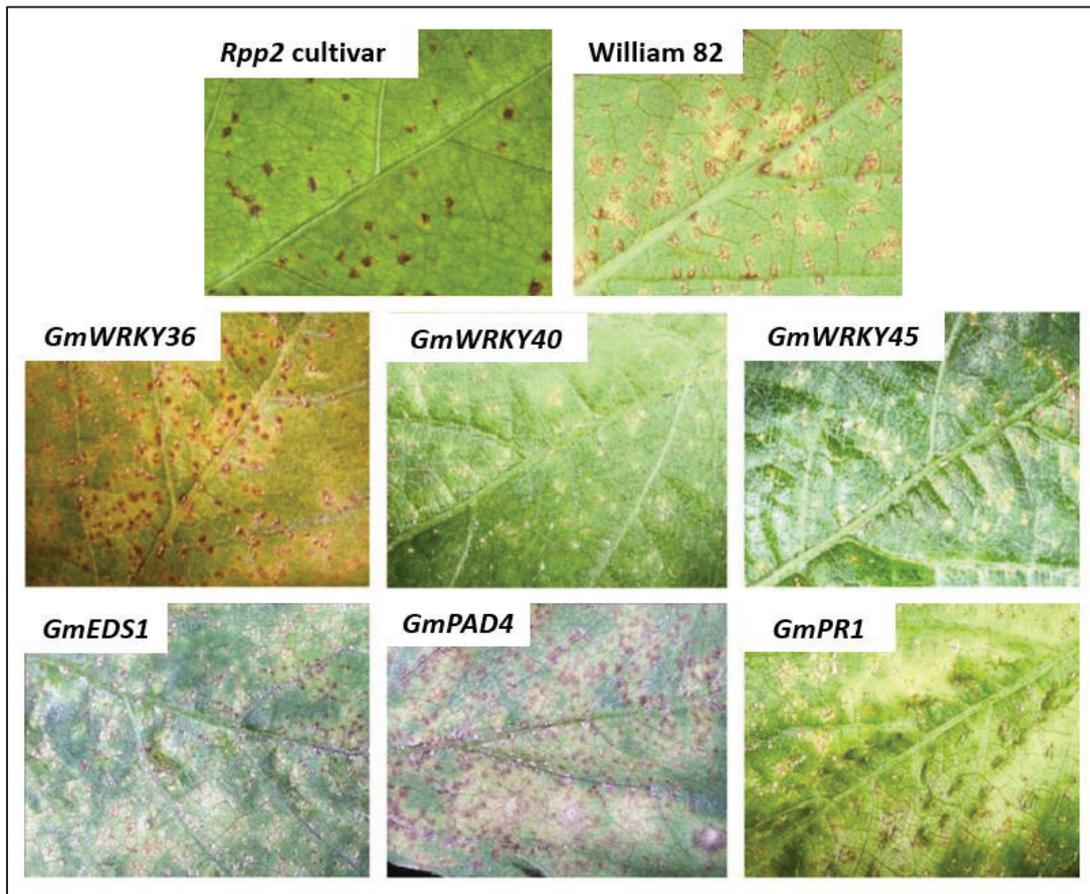


Figure 22: **Loss of *Rpp2*-mediated resistance to *P. pachyrhizi* following silencing of different genes**  
 Silencing was realized by virus-induced gene silencing (VIGS). Silenced *Rpp2* plants were inoculated 3 weeks after virus treatment, and leaves were pictured 2 weeks later. Loss of resistance was observed when *GmEDS1*, *GmPAD4*, *GmNPR1*, *GmWRKY36*, *GmWRKY40*, *GmWRKY45* and *GmO-MT* were silenced. The treatment of *Rpp2* plants with the virus without insertion did not affect the RB phenotype. Infected Williams 82 plants showing susceptible TAN lesions served as control. From Pandey *et al.*, 2011.

### **(8) Engineering cell death**

One strategy to reduce *P. pachyrhizi* penetration success in barley has been achieved by cell death suppression. Barley is penetrated by *P. pachyrhizi*, resulting in epidermal cell death and cell wall structures apposition (such as callose). However, the dead epidermal cells did not generally restrict the fungal development but allowed a partial mesophyll invasion. Expression of the barley cell death suppressor *BI-1* reduced both epidermal cell death and fungal penetration success (Hoefle *et al.*, 2009). This finding suggests that *P. pachyrhizi* induced programmed cell death to facilitate its entry into epidermal cells of barley. However, once in barley mesophyll, no haustoria are formed and mesophyll cell death occurs, stopping the fungal growth. This outcome indicates that once in the mesophyll, *P. pachyrhizi* exhibits a biotrophic life style as typical rust fungus (Hoefle *et al.*, 2009).

Another method to develop transgenic plants with increased disease resistance is to produce an artificial “HR-like” response. The success of this strategy is based on the rapid generation of an artificial cell death localized only at the infection sites (Mourgues *et al.*, 1998). This strategy has never been used to control Asian soybean rust. The HR is characterized by rapid and localized death of plant tissues at the infection site and activation of biochemical processes. This results in the formation of lesions, where the pathogen development is restricted (Greenberg, 1997). To better explain this mechanism, the next chapter will focus first on the HR and methods to induce cell death.

#### 4) Cell death induction

##### The hypersensitive response

HR is a specific form of programmed cell death (PCD). PCD is an essential process involved in embryo development, ageing, senescence, cell differentiation and immunity (Mukhtar *et al.*, 2016). In plants, PCD is classified into two groups: apoptosis and necroptosis (Figure 23a-b). Apoptosis is characterized by protoplast shrinkage, vacuole swelling, organelle degradation and nuclear fragmentation. Necroptosis (self-eating) occurs with protoplast rupture and shrinkage and organelle swelling. During the HR, protoplast shrinkage, vacuole and organelle swelling and nuclear fragmentation are observed (Figure 23-c). Consequently, the HR is considered a hybrid form of cell death sharing characteristics of apoptosis and necroptosis PCD (Mukhtar *et al.*, 2016).

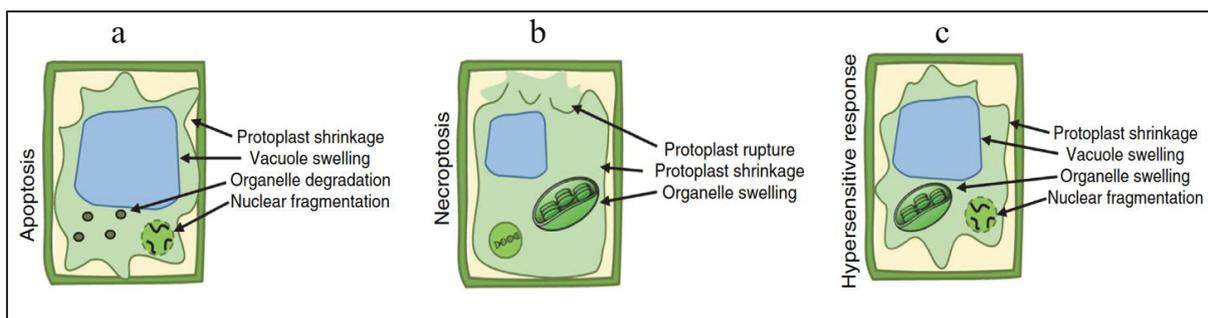


Figure 23: **Comparison of cell death morphology in plants** (a) Apoptosis, (b) necroptosis and (c) hypersensitive response (HR). From Mukhtar *et al.*, 2016.

In addition to a PCD mechanism, different biochemical processes are also associated with the HR. Reactive oxygen species (ROS) are massively produced. This leads to structural decomposition and permeability changes of the cell membrane in addition to damage to essential proteins and DNA. The intracellular calcium level is modified as the result of the mediated-cell suicide signal (Iakimova *et al.*, 2005). Antimicrobial compounds are also

released, salicylic acid accumulation is observed, and transcriptional reprogramming including defence-related genes induction occurs (Mur *et al.*, 2008). As noted above (see soybean reaction to *P. pachyrhizi* infection), resistance to Asian soybean rust is associated with a hypersensitive response. In RB lesions, multicellular cell death of epidermal and mesophyll cells restricts the development of the pathogen.

### **Strategies to induce cell death**

HR is particularly efficient against biotrophic fungi and therefore appears as an interesting potential strategy for soybean rust control. Different approaches have been proposed to induce local cell death without interfering with the natural and highly complex plant defence mechanisms. Transgenic tobacco plants expressing an avirulence gene (*avr9*) from *Phytophthora cryptogea* under the control of a pathogen-inducible promoter (*hsr203j* promoter from *Nicotiana tabacum*) have been developed (Islam, 2006; Keller *et al.*, 1999). Those plants exhibit a restricted HR response following inoculation with *Phytophthora nicotianae*. According to the same concept, a two-component system could be imagined with the expression of an avirulence gene under the control of a pathogen-inducible promoter and the corresponding *R* gene under the control of a constitutive promoter (Mourgues *et al.*, 1998).

Another approach includes a system based on the expression of barnase and barstar bacterial genes (Strittmatter *et al.*, 1995). In 1995, Strittmatter *et al.* developed transgenic potatoes resistant to *P. infestans* by engineering controlled cell death. They selected the barnase and barstar genes from *Bacillus amyloliquefaciens*. The barnase gene encodes a ribonuclease (RNase) (110 amino acids), and the barstar protein (89 amino acids) is a highly specific barnase inhibitor. It is proposed that the barnase serves the organism by degrading RNA in its environment for nutritional use, while the barstar protects *B. amyloliquefaciens* from the toxic effects of intracellular barnase activity (Hartley, 1989). Barstar is able to form a highly stringent complex with the barnase in which the barnase activity site is covered, leading to its inactivation (Hartley, 2001). Strittmatter *et al.* placed the barnase gene under the control of a pathogen-inducible promoter. To express the barnase only in the presence of *P. infestans*, at the infection site, the authors selected the *P. infestans*-inducible promoter *pgst1* (Martini *et al.*, 1993) from *Solanum tuberosum*. To bypass the potential background activity of the pathogen-inducible promoter in non-infected tissues, the barstar gene was placed under the 35S constitutive promoter from the cauliflower mosaic virus to counter-balance the barnase expression. Thus, without infection, the barnase background should be inhibited by the barstar expression (Figure 24-a), while under pathogen attack, the barnase/barstar ratio should be in favour of the barnase, leading to the collapse of the cells (Figure 24-b).

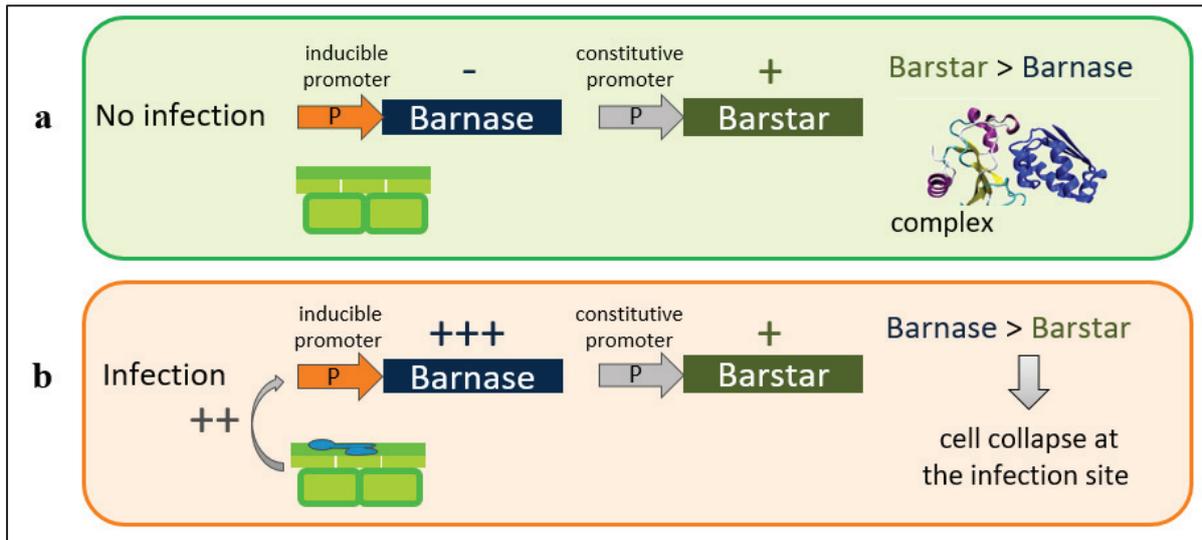


Figure 24: **Barnase/barstar system** a) Without infection, the barnase quantity in the plant cell is very low and the barstar expression is sufficient to cover the barnase background. b) In the presence of the pathogen, the barnase promoter is induced and the quantity of barnase becomes dominant, leading to cell collapse at the infection point.

This system allowed the development of transgenic potatoes (*Solanum tuberosum*) showing a diminution in sporangia formation from 33 to 87% five days post-inoculation with *P. infestans*. The phenotype of those plants was similar to that of WT potato (Strittmatter *et al.*, 1995). The strategy was also successfully used for the development of artificial male sterility in different commercial crops by using tissue-specific promoters (Mariani *et al.*, 1990).

## 5) Conclusion

Current knowledge of plant/pathogen interaction highlights a plethora of genes implicated in plant defence responses. Different genes show potential for the development of soybean lines resistant to *P. pachyrhizi*, such as the *R* gene from *Cajanus cajan* *CcRpp* (Kawashima *et al.*, 2016). However, a single gene may be risky in the medium to long term, and it would be prudent to develop soybeans with several resistance genes that have different mechanisms or specificities (Kawashima *et al.*, 2016). Currently, there is no report on cell death induction for engineering *P. pachyrhizi* resistance in this work. The barnase/barstar system allowing artificial cell death will be evaluated as a new tool for the development of transgenic soybeans resistant to *P. pachyrhizi*. To this end, a promoter inducible by *P. pachyrhizi* is necessary for the control of *barnase* expression. .

## E. PROMOTERS FOR PLANT DISEASE ENGINEERING

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The study of plant gene promoters is central for understanding the global regulation of gene expression. Such research also contributes to extending the toolbox of available promoters for use in plant biotechnology approaches (Hernandez-Garcia and Finer, 2014).

### 1) Promoter structure

The promoter corresponds to the DNA region upstream of the transcription start site (TSS) of a gene (Hernandez-Garcia and Finer, 2014). The promoter contains the recognition site for polymerase (type II for messenger RNA synthesis) and various sequence elements. Distal regulatory elements can be localized near the TSS or far away, either upstream or downstream of the gene they regulate. These elements include enhancers (positive transcription regulators), silencers (negative transcription regulators) and insulators (preventing promiscuous gene regulation) (Figure 25-a) (Yadav *et al.*, 2016). The promoter can be divided into two regions: the core and the proximal promoter. The core promoter includes 100 bp around the TSS and includes the TATA box (~35 bp upstream of the TSS), which is the binding site for transcription initiation factors and the RNA polymerase II (Bilas *et al.*, 2016). Another element frequently observed is the CAAT box (~80 bp upstream the TSS) that can influence the expression efficiency. In plants, instead of the CAAT box, a similar AGGA box has been identified (Bilas *et al.*, 2016). The proximal promoter is located downstream of the core promoter. This promoter includes cis-regulatory elements that contain binding sites for transcription factors and/or regulatory proteins. These elements also contribute to the fine regulation of the gene expression (Figure 25-b) (Muthusamy *et al.*, 2017).

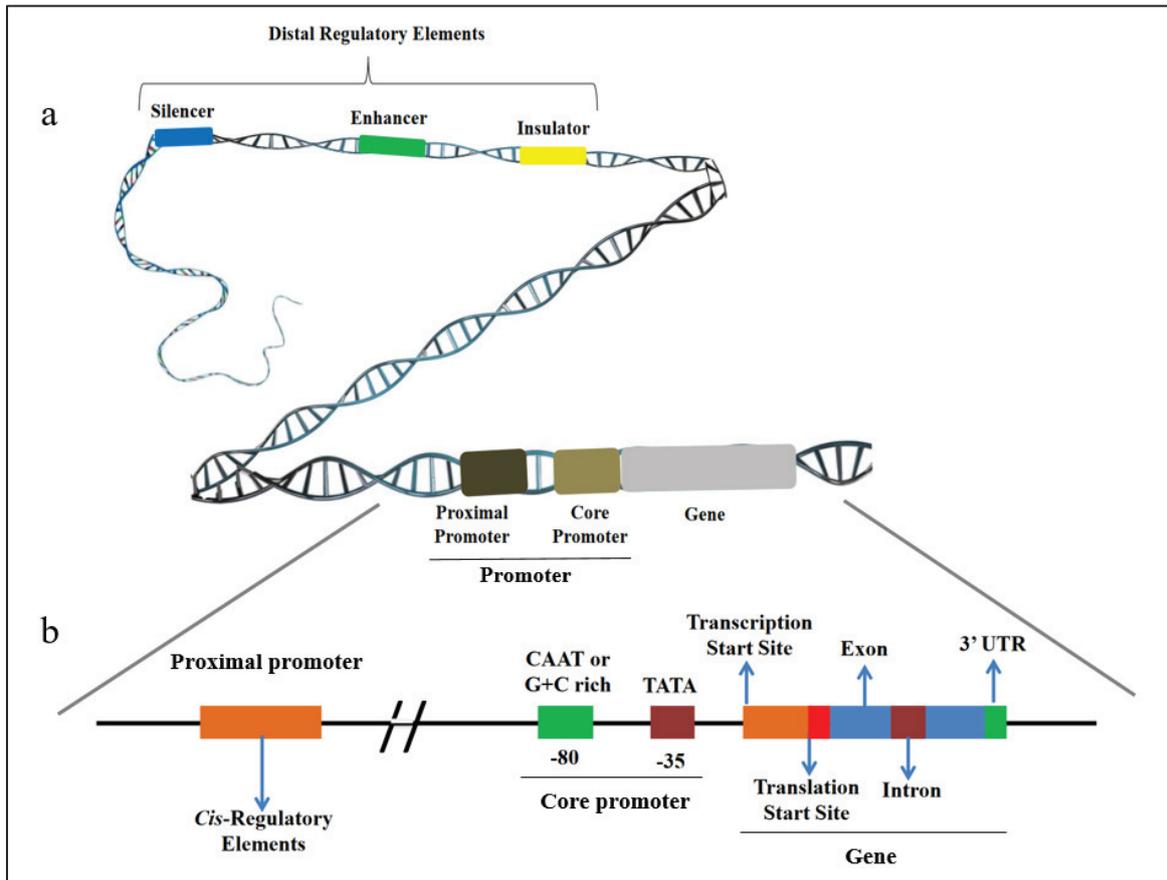


Figure 25: **Transcriptional regulatory elements in plants** a) Schematic representation of gene regulatory regions. b) Typical promoter elements found in eukaryotes. Adapted from Yadav *et al.*, 2016.

Certain promoters are active in all cell types and developmental stages (constitutive promoters), whereas others are specifically expressed in specific plant tissues (tissue specific promoters) or require a stimulus for activation (inducible promoters). This section focuses only on constitutive and pathogen-inducible promoters that will be used in this study.

## 2) Constitutive promoters

Constitutive promoters allow the expression of a gene in most plant tissues under any conditions. A common strategy in plant disease engineering is to overexpress a selected gene under the control of a constitutive promoter to evaluate its potential to enhance resistance (Gurr and Rushton, 2005b). To this end, the well-studied 35S promoter from the cauliflower mosaic virus (CaMV) has been extensively used.

### The 35S promoter

This viral promoter that drives the expression of the CaMV ribosome 35S unit has shown to allow a high expression in different tissues of many plants (Jefferson *et al.*, 1987; Williamson

*et al.*, 1989). However, it has been shown to be more effective in dicots than in monocots (Bilas *et al.*, 2016). The 35S promoter contains two main domains: A and B (Figure 26). Domain A is composed of subdomain A1 and the minimal promoter part (region containing the TATA box). Domain A only exhibited a high expression mainly in roots. In contrast, domain B was subdivided into five subdomains: B1 to B5. The individual subdomains of domain B could drive specific spatial expression in addition to domain A. It was proposed that expression of the 35S promoter is the result of a combinatorial effect of the various subdomains (Bhullar *et al.*, 2007). However, the expression driven by the 35S promoter is often lower in reproductive tissues. Nevertheless, the 35S promoter is present in most of the transgenic soybean events on the market, including, for instance, Roundup Ready and *Bt* plants respectively resistant to an herbicide and lepidopterans (Porto *et al.*, 2014) (see soybean production).



Figure 26: **Organization of the CaMV35S promoter.** mp: minimal promoter (region containing the TATA box). From Benfey and Chua 1990.

### Other constitutive promoters

Considering the results obtained with the CaMV35S promoter, different viral promoters have been studied for their use in plant engineering. For instance, a promoter fragment from the cassava vein mosaic virus (CsVMV) was identified as driving a high expression level in plants (Verdaguer *et al.*, 1996). The constitutive pattern of expression of this promoter was attributed to distinct elements driving organ-specific expression (Verdaguer *et al.*, 1998). A higher GUS expression was observed in transgenic alfalfa (*Medicago sativa*) plants with the CsVMV promoter than in plants with the 35S promoter (Figure 27). Therefore, in alfalfa the CsVMV promoter has been shown to allow a higher expression level than the 35S promoter (Samac *et al.*, 2004).

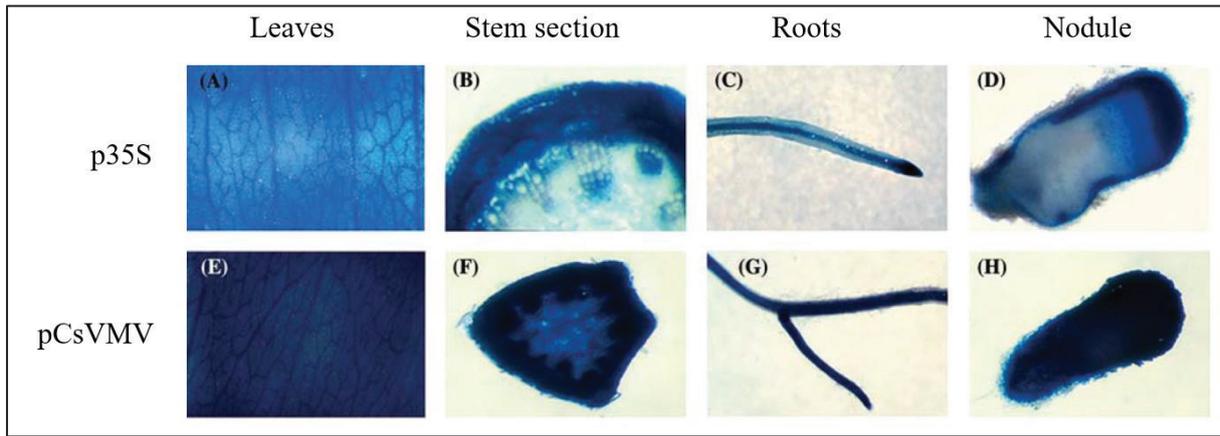


Figure 27: Expression in *Medicago sativa* of the CaMV35S and the CsVMV promoters. GUS detection in plants containing the p35S:GUS (A-D) and the pCsVMV:GUS transgenes (E-H). Adapted from Samac *et al.*, 2004.

Constitutive promoters used in plant engineering have also been isolated from bacteria. For example, the *Agrobacterium tumefaciens* NOS promoter controls the expression of the nopaline synthase gene. The NOS promoter has been mainly used for selectable marker expression in transgenic plants (Fraley *et al.*, 1983). The precise activity of this promoter was studied in tobacco, revealing an organ-specific and developmental regulation. The promoter expression was high in the lower part of the plant and in various flower organs but low in upper plant parts and in vegetative organs (An *et al.*, 1988). These observations demonstrated that the activity of a potential constitutive promoter may be subject to variations. Nevertheless, the NOS promoter has been reported as providing a lower expression than the 35S promoter (Sanders *et al.*, 1987). In addition, many efficient constitutive promoters have been isolated from plants. The high expression of plant housekeeping genes that encode for proteins required for basic functions in cells (e.g., ubiquitin actin or tubulin) indicates good source for strong native constitutive promoters (Hernandez-Garcia and Finer, 2014). In the case of monocotyledon plant engineering, a promoter from maize controlling a ubiquitin (*ZmUbi1*) is widely used to allow constitutive expression. However, the *pZmUbi1* activity tends to decrease with plant maturation showing variations, as was also observed for the NOS promoter (Park *et al.*, 2010). In soybean, Hernandez-Garcia *et al.* evaluated the activity of 20 promoters from two different gene families: ubiquitin (*Gmubi1-10*) and Ethylene Response Factor (*GmERF1-10*) (Hernandez-Garcia *et al.*, 2010). The expression of these promoters was compared to that of the 35S promoter after transient tissue transformation in hairy roots of soybeans. To this end, the GFP gene was expressed under the control of different promoters, and the tissue fluorescence of the resulting transformed plants was analysed. Although the results were not exactly the same

depending on the transformation technique used, the authors highlighted several promoters with lower and higher activity than the p35S (Table 4).

Table 4: **Grouping of the *Gmubi* and *GmERF* promoters based on the 35S-driven GFP expression.** From Hernandez-Garcia *et al.*, 2010.

Promoter	Transient GFP expression			GFP expression in hairy roots		
	Low	Moderate	High	Low	Moderate	High
Gmubi #	10	2, 8	1, 3, 4, 5, 6, 7, 9	10	5, 6, 8	1, 2, 3, 4, 7, 9
GmERF #	1, 8, 9	2, 4, 7	3, 5, 6, 10	1, 5, 8, 9	3, 4, 7	2, 6, 10

GFP levels driven by the CaMV35S promoter in both transient and hairy roots were considered as moderate.

A large number of constitutive promoters are available to control gene expression. Additionally, the expression of toxic proteins must be strictly controlled. Furthermore, permanent and high ectopic expression of defence-related genes can impact the plant's fitness and development (Gurr and Rushton, 2005a). For instance, expression of one or more *R* genes can reduce plant fitness. This effect was observed with the expression of an *Arabidopsis* *R* gene (RPM1 coding for a peripheral plasma membrane protein) in a susceptible *A. thaliana* ecotype that induced a reduction in seed number and shoot biomass (Tian *et al.*, 2003). These challenges can be overcome by using an inducible promoter that allows transgene expression only when and where it is needed. Promoters induced in response to pathogen infection can help address these challenges.

### 3) Pathogen inducible promoters

The advantage of pathogen-inducible promoters is well illustrated by the expression of a multi-pathogen-resistant gene (*Lr34res*) in barley (Boni *et al.*, 2018; Chauhan *et al.*, 2015; Risk *et al.*, 2013). The *Lr34res* gene was originally identified in wheat as providing durable resistance to three wheat rusts (*Puccinia triticina*, *Puccinia striiformis* and *Puccinia graminis*) and powdery mildew (*Blumeria graminis* f.sp. *tritici*). This gene encoding an ATP-binding cassette (ABC) transporter was successfully transferred in barley and conferred resistance against leaf rust *Puccinia hordei* and the powdery mildew *Blumeria graminis* f.sp. *hordei*. However, *Lr34res* expression under its native promoter resulted in pleiotropic effects on growth and fitness as well as a strong phenotype of leaf tip necrosis on barley plants (Risk *et al.*, 2013). To

avoid those pleiotropic effects, Boni *et al.* (2018) developed transgenic barley expressing the *Lr34res* gene under control of the well-described barley germin-like GER4 promoter. This promoter has been shown to be induced by pathogens (*Blumeria graminis* f.sp. *hordei* and *Rhynchosporium secalis*) (Himmelbach *et al.*, 2010). GUS staining of transgenic barley plants expressing the GER4 promoter fused to the GUS reporter gene showed only a local induction of the promoter with *Bgh* pathogen (Figure 28). In the GMO containing the GER4 promoter *Lr34res* cassettes, a reduction of symptom following rust and mildew infection was observed (Figure 29-a). The negative pleiotropic effects were reduced with decreased leaf tip necrosis in the case of barley with the *Lr34res* gene under control of the pathogen-inducible promoter compared to barley plants with the same gene under control of its native promoter (Figure 29-b) (Boni *et al.*, 2018).



**Figure 28: Local induction of the GER4 promoter by the *Blumeria graminis* f.sp. *hordei* pathogen** (a) A leaf segment from a transgenic T1 barley carrying the GER4 promoter fused to the GUS reporter gene was covered with a sheet containing the cutout letters MILDEW, followed by inoculation with *Blumeria graminis* f.sp. *hordei*. GUS staining 24h after inoculation. (b) Microscopy analysis of GER4 promoter activity, revealed 24 h after *Blumeria graminis* f.sp. *hordei* inoculation using histochemical GUS staining. Germinated fungal spore in dark-blue. Bar = 50 mm. Adapted from Himmelbach *et al.*, 2010.

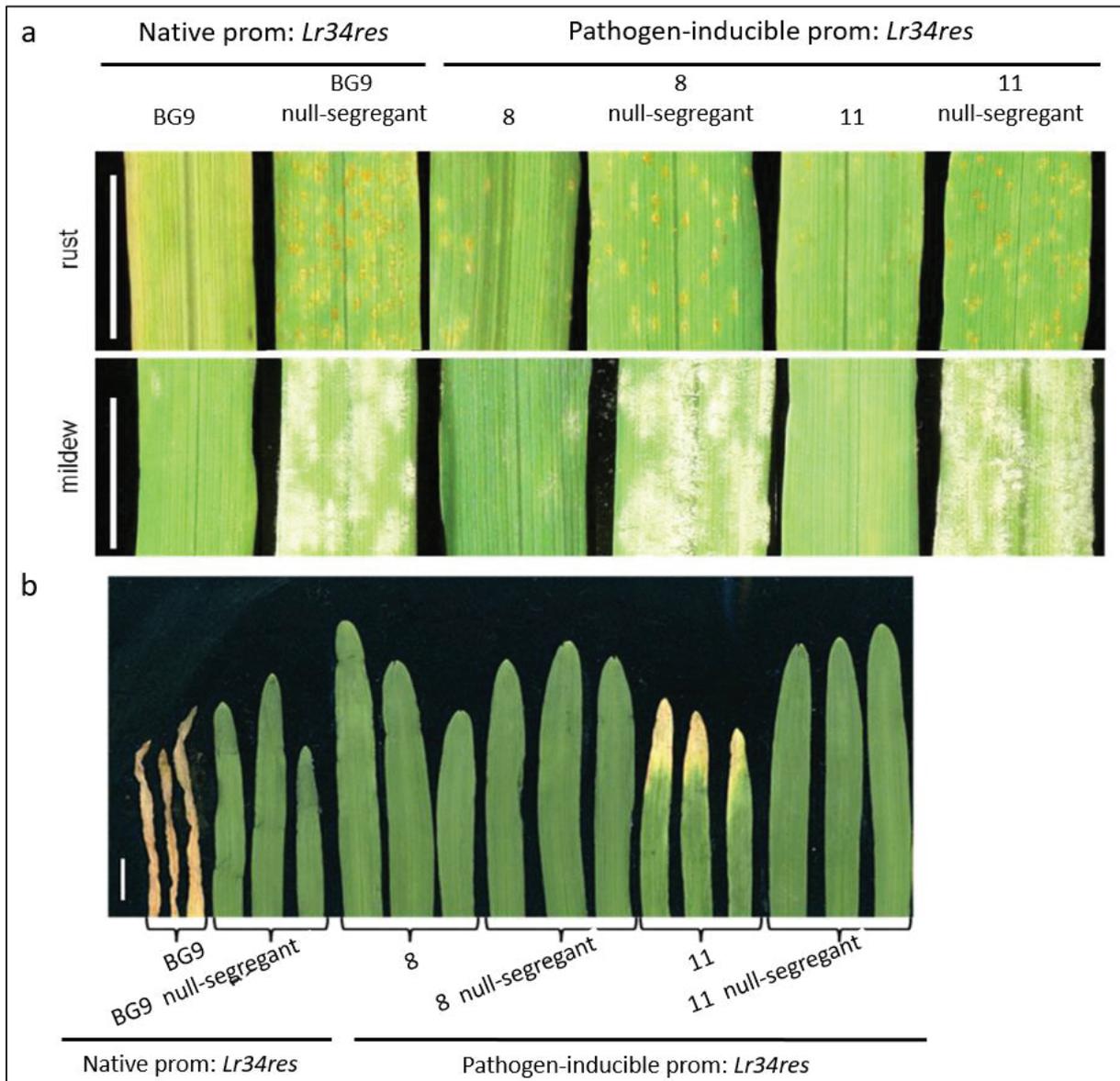


Figure 29: **Pathogen-inducible expression of *Lr34res* in barley.** a) The pathogen-inducible promoter *Lr34res* transgenic barley shows *Lr34res*-mediated disease resistance (the fourth leaves of plants at the five-leaf stage were taken 7 dpi). b) The barley lines expressing *Lr34res* under control of a pathogen-inducible promoter show reduced leaf tip necrosis and reduced impact on growth parameters. Adapted from Boni *et al.*, 2018.

Pathogen-inducible promoters have been communally isolated from genes associated with defence response in plants (Hernandez-Garcia and Finer, 2014). For example, the barley germin-like GER4 promoter controls the expression of a PR protein (Himmelbach *et al.*, 2010). These promoters contain cis-regulatory elements that can be activated by different stresses (Muthusamy *et al.*, 2017). Consequently, pathogen-inducible promoters are often induced by other stimuli such as wounding and/or hormones.

Many pathogen-inducible promoters have been identified from different plants such as *Arabidopsis*, tobacco and rice (Himmelbach *et al.*, 2010; Manners *et al.*, 1998; Yang *et al.*,

2017). However, few pathogen-inducible promoters have been reported in soybean. A soybean promoter controlling the expression of a calmodulin isoform, *GmCaM-4*, was shown to be triggered by *P. syringae* pv *tomato* and NaCl in tobacco (Park, 2004). This finding demonstrates the often-observed non-specificity of pathogen-inducible promoters. Two soybean promoters controlling a polyphenol oxidase (GmPPO12) and an enzyme functioning as a protease inhibitor (GmSKTI36), have been found to be rapidly and strongly induced by the fungal pathogen *Phytophthora sojae* in transiently transformed soybean hairy roots (Chai *et al.*, 2013, 2016). Both of these genes were identified through the investigation of microarray data analysing the soybean response to *P. sojae*.

#### 4) Conclusion

Many genes associated with disease resistance have been identified and proposed to develop transgenic plants to fight pathogens (Ali *et al.*, 2017; Gurr and Rushton, 2005a; Silva *et al.*, 2018). Generally, these genes have been expressed under well-known constitutive promoters and the CaMV 35S promoter was extensively used for this purpose. Nevertheless, many other promoters suitable to drive constitutive expression have been discovered. However, constant expression of such genes can have negative effects on the plant and is not necessary in the absence of the pathogen. To drive the expression of disease resistance genes only in cases needed, i.e., pathogen infection, pathogen-inducible promoters are required. Such promoters have been isolated in several plants mainly from genes associated with defence response (Smirnova and Kochetov, 2015). Nevertheless, the identification and characterization of such promoters in soybean is still limited (Chai *et al.*, 2013; Hernandez-Garcia and Finer, 2016). In biotechnology methods developed for soybean rust resistance, a promoter induced by *P. pachyrhizi* has never been used, revealing a lack of information in this area.

## F. OBJECTIVES

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*Phakopsora pachyrhizi* is a biotrophic fungal pathogen responsible for the Asian soybean rust disease causing important yield losses in tropical and subtropical soybean-producing countries. Today, the control of this pathogen is based on fungicidal treatments. However, a decrease in treatment efficacy of some molecules has been observed. The dominant *R* genes identified are not seen as a complementary approach to the use of pesticides. They are only effective against specific isolates of *P. pachyrhizi* and can be overcome by the fungus. In this context, biotechnological approaches offer alternative solutions to control the Asian soybean rust disease.

The current project is oriented on the development of a new biotechnology method for *P. pachyrhizi* control: engineering cell death. This strategy is based on the induction of a rapid plant cell death localized around the infection sites when the plant is attacked by the pathogen. Cell death will be triggered by the barnase/barstar system, in which barnase gene expression is toxic for the cell and the barstar protein inhibits the barnase activity. To restrict plant cell death, it is proposed to place the barnase under the control of a promoter inducible by *P. pachyrhizi*. Barstar will be placed under the control of a constitutive promoter to cover any possible background expression of barnase.

To this end, a promoter inducible by the soybean rust is mandatory. Consequently, the work is divided into 3 parts (Figure 30). 1) Candidate promoters potentially inducible by *P. pachyrhizi* will be identified using transcriptomic and bibliographic data. 2) The candidate promoters will be evaluated *via* GFP reporter gene expression. Their activity will be assessed under pathogen infection. For the promoters showing an induction by *P. pachyrhizi*, their profiles in response to other stresses (wounding and hormonal treatments) will be observed. 3) The best candidates will be used in the cell death technology for the evaluation of the technology against *P. pachyrhizi*.

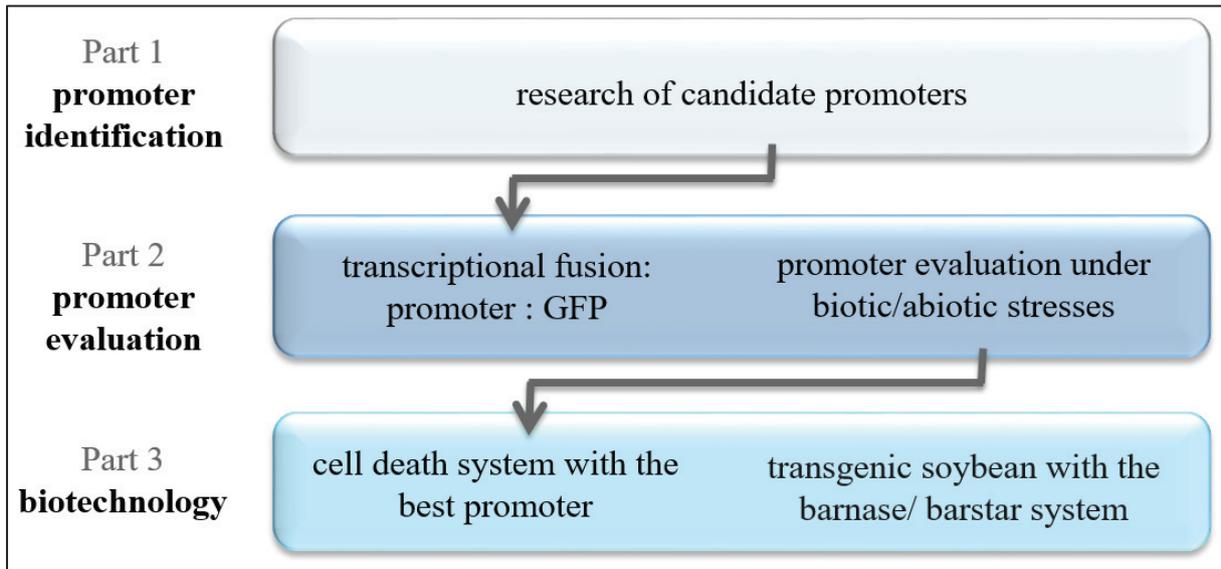


Figure 30: Workflow



# MATERIALS AND METHODS

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All the experiments were conducted according to the recommendations of the French biosafety agency (Haut Conseil des Biotechnologies).

## A. - BIOLOGICAL MATERIAL

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### 1) Eukaryotic biological material

#### Soybean

The commercial soybean variety “Thorne” (*Glycine max* (L.) Merr.) was originally provided by Ohio State University (Laboratory of J. Finer, Wooster, OH, USA). The seed lot was tested for the absence of GM traits (bar, epsps, 35S and NOS elements). Seeds were increased in the field either in Argentina or the USA under containment conditions to preserve identity. Seeds were sown in 7x7 cm pots containing SteckMedium substrate (Klasmann-Deilmann GmbH, Germany) for germination. After 3 weeks, plants were transferred to a larger pot for development and eventually seed production. Greenhouse conditions were as follows: temperature of 24 °C day/22 °C night with a photoperiod of 16 h of day under a light intensity of 270  $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  and 70% relative humidity. The plants were watered three times by week with 1% fertilizer (Algospeed Flo 15.15.15).

Another soybean variety, “Jack”, also provided by Ohio State University, was used for transcriptomic analysis. Conditions of production and cultivation were similar to those of Thorne.

#### “Tobacco”

Seeds of *Nicotiana benthamiana* were sown in a 20x30 cm tray (SteckMedium substrate). After 3 weeks, young plants were transferred to individual pots for additional development for 2 weeks. Greenhouse conditions were as follows: temperature of 26 °C day/24 °C night, 16 h of day under a light intensity of 270  $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  and 50% relative humidity. The plants were watered three times by week with 1% fertilizer (Algospeed Flo 15.15.15).

#### Soybean rust

The original inoculum of *Phakopsora pachyrhizi* Syd. & P. Syd. was sampled as a bulk in a field in the state of Mato Grosso (Brazil) in 2006. The samples of spores were sent to Europe and are currently routinely used for research purposes. The spores were multiplied on susceptible soybeans, harvested with a paint brush, filtered on a screen to eliminate trichomes,

aliquoted in 20 mg samples and finally dehydrated before being stored in liquid nitrogen. The frozen spores stored in liquid nitrogen were used as a routine source of inoculum.

## 2) Prokaryotic biological material

### *Escherichia coli*

Library Efficiency® DH5 $\alpha$ <sup>TM</sup> Competent Cells (INVITROGEN) were used for transformation, multiplication and plasmid DNA extraction. DH5 $\alpha$  Competent Cells possess a transformation efficiency of  $1 \times 10^7$ - $1 \times 10^8$  transformants/ $\mu$ g. The genotype of DH5 $\alpha$  is as follows: *F*<sup>-</sup>,  $\Phi$ 80*d lacZ*  $\Delta$ *M15*,  $\Delta$  (*lacZYA-argF*) *U169*, *deoR*, *recA1*, *endA1*, *hsdR17* (*rk*<sup>-</sup>, *mk*<sup>+</sup>), *phoA*, *supE44*,  $\lambda$ <sup>-</sup>, *thi-1*, *gyrA96*, and *relA1*. Stellar strain (thermo-competent) (CLONTECH) was used for transformation of an InFusion reaction product when it was difficult to obtain a bacterial colony after DH5 $\alpha$  transformation. Stellar bacteria possess a transformation efficiency greater than  $1 \times 10^8$  transformants/ $\mu$ g ( $5 \times 10^8$  transformants/ $\mu$ g), which is recommended for InFusion cloning. Stellar strain genotype is as follows: *F*<sup>-</sup>, *endA1*, *supE44*, *thi-1*, *recA1*, *relA1*, *gyrA96*, *phoA*,  $\Phi$ 80*d lacZ*  $\Delta$  *M15*,  $\Delta$  (*lacZYA-argF*) *U169*,  $\Delta$  (*mrr-hsdRMS-mcrBC*),  $\Delta$ *mcrA*,  $\lambda$ <sup>-</sup>. Both of these strains are suitable for screening on X-Gla or Bluo-Gla ( $\Phi$ 80*d lacZ*  $\Delta$ *M15*), growth on minimal media (*deoR*), high-quality plasmid DNA preparations (*endA1*) and cloning with stabilization of vector carrying a cloned insert (*recA1*) and no deletion between direct repeats (*gyrA96*). The *E. coli* cultures were grown at 37 °C for 24 h, and liquid cultures were agitated at 180 rpm.

### *Agrobacterium tumefaciens*

ElectroMAX<sup>TM</sup> *Agrobacterium tumefaciens* LBA4404 Cells (INVITROGEN) were used for soybean stable transformation and tobacco transient transformation. The LBA4404 strain (octopine-type) contains the disarmed Ti plasmid pAL4404 (with only the *vir* and *ori* regions) and a rifampicin (chromosomal) and streptomycin (on the Ti plasmid) resistance (Hellens *et al.*, 2000). The EHA105 strain was used for transient transformation of soybean. EHA105 strain (agropine-type) contains the disarmed Ti plasmide pEHA105 (pTiBo542  $\Delta$ T-DNA) and a rifampicin (chromosomal) resistance (Hellens *et al.*, 2000). *A. tumefaciens* cultures were grown at 28 °C for 48 h, and liquid cultures were agitated at 180 rpm.

Bacterial cultures were grown on solid or liquid *lysogeny broth* (Lb) medium (15 g/L agar, 10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl) (Q-BIOGENE) with the appropriate antibiotics (100 mg/L spectinomycin, 50 mg/L kanamycin or 100 mg/L carbomicilin).

### 3) Soybean samples used for RNA sequencing

Three week-old soybean plants of the cultivar "Jack" susceptible to *P. pachyrhizi* were used. After rehydration (one night at 21°C in the dark), the spores of *P. pachyrhizi* were suspended for 30 min in sterilized water containing 0.01% Tween 20 to reach a final concentration of 100,000 spores/mL. Soybean leaves were sprayed with this inoculum solution of 100,000 spores/mL in 0.01% Tween 20 or a mock solution of 0.01% Tween 20 until run-off. The plants were incubated in a growth chamber (temperature 24 °C, dark, 100% relative humidity) for 24 h.

Another set of plants were sprayed with 200 ppm of chitin oligosaccharides (the chitinheptaose DP7) or water until run-off. The plants were incubated in a growth chamber (temperature 24 °C, 16 h light/8 h dark, light intensity 15  $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  and 80% relative humidity) for 24 hours.

Leaf samples were harvested at 0, 0.5, 1, 2, and 24 hours post infection or DP7 treatment. RNA extraction was conducted as described in nucleic acid extraction part, section plant RNA.

## B. MOLECULAR BIOLOGY

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### 1) Nucleic acid extraction

#### Plasmid DNA

Plasmid DNA extraction was used for different purposes such as cloning, verification of bacterial clones and transformation of bacteria.

An overnight bacterial culture (from a glycerol stock) of 2 mL was used to recover 60 to 200 ng of plasmid DNA. When more DNA was needed, the bacterial cultures were adjusted (2 x 2 mL for 400 ng of DNA for example). The bacterial culture was centrifuged for 10 min to pellet the bacteria. The supernatant was discarded and the plasmid DNA extraction was performed with the QIAprep® Spin Miniprep kit (QIAGEN). The pelleted bacteria were diluted in 250 µL of resuspension P1 buffer (50 mM Tris-HCl, 10 mM EDTA, pH 8 and RNase A 100 µg/mL). A total of 250 µL of P2 buffer (200 mM NaOH and 1% SD) allowing an alkaline lyse of the bacteria and 350 µL of N3 buffer (3 M potassium acetate, pH 5.5) for lysis reaction neutralization were added to the sample. After 10 min of centrifugation, the supernatant containing the plasmid DNA in a solution of high salt concentration was loaded into a QIAprep spin column. This column possesses a silica membrane allowing the DNA binding. A centrifugation step of 1 min was performed, and the flow-through was discarded. The column was washed with a PB solution (500 µL) allowing endonuclease to be removed and with the PE buffer containing ethanol (750 µL) for salt removal. Each step was followed by a centrifugation step of 1 min. The flow-through was discarded, and an additional 2 min centrifugation step was performed to clear the column from the remaining buffer solution. The plasmid DNA was eluted with 50 µL of elution buffer (10 mM Tris-HCl, pH 8.5). All the centrifugations were performed at 10,000 g.

#### Plant DNA

Plant DNA extractions were conducted in order to analyse by ddPCR the number of inserts of the *HPPD-4* gene (4-hydroxyphenylpyruvate dioxygenase gene of *Pseudomonas fluorescens*) selectable marker transferred by *A. tumefaciens* in transgenic plants.

For one sample, four foliar discs of 1 cm were harvested in a tube containing one stainless steel ball (5 mm) for tissue disruption and placed at -80 °C. The tissues were crushed using a Mixer Mill MN3000 (RETSCH). Total genomic DNA extractions were achieved by using the

DNeasy® Plant Mini kit (QIAGEN). The lysis buffer AP1 (400 µL) and RNase A (4 µL) were added to the disrupted material. The samples were incubated 10 min at 65 °C for tissue lysis. The samples were placed for 5 min on ice after the addition of 130 µL of P3 solution for precipitation of proteins and polysaccharides. After 5 min of centrifugation, the lysate was placed into a QIAshredder spin column to remove the cell debris and the precipitates. Following 2 min of centrifugation, the flow-through was transferred to a new tube and 1.5 volume of AW1 buffer (containing ethanol) was mixed with the sample to allow DNA binding to the DNeasy membrane. A total of 650 µL of the mixt was placed in a DNeasy Mini spin column and was centrifuged for 1 min. The flow-through was discarded, and this step was repeated with the remaining sample to load the total DNA on the column. After the addition of 500 µL of buffer AW2, the spin column was centrifuged for 1 min to clear the column of any contaminant. The plant DNA elution was performed with 100 µL of elution buffer (10 mM Tris·Cl, 0.5 mM EDTA, pH 9.0). All the centrifugations were performed at 10,000 g.

### **Plant RNA**

Plant RNA was extracted for RT-qPCR reactions to follow soybean gene expression under infection by *P. pachyrhizi* and salicylic acid treatment.

Four foliar discs of 1 cm were harvested per sample in a tube containing one stainless steel ball (5 mm) for tissue disruption. The samples were directly placed first in liquid nitrogen and then stored at -80 °C. The tissues were crushed using the Mixer Mill MN3000 (RETSCH). Total RNA was extracted by using the RNeasy® Plant Mini Kit (QIAGEN). The buffer RLT (450 µL), containing β-mercaptoethanol, was added to the samples for the lysis of tissues and the RNase inactivation. The solution was vortexed for 3 min, and the lysate was transferred in a QIAshredder spin column and centrifuged for 2 min to remove insoluble material and reduce the viscosity of the lysate. A 0.5 volume of ethanol (96-100%) was added to the supernatant to create conditions for selective binding of RNA to the RNeasy membrane. The solution was immediately mixed and placed in an RNeasy Mini spin column for a centrifugation step of 1 min. To remove any contaminants, the column was washed with 700 µL of buffer RW1 and twice with 500 µL of buffer RPE with a step of centrifugation after the addition of each buffer. The RNA elution was realized with 30 µL of RNase-free water. Extracted RNA was treated with TURBO DNA-free™ Kit (INVITROGEN). Volumes of 0.1 µL of Turbo DNase buffer (10X) and 1 µL of Turbo DNase were added, and the solution was incubated 30 min at 37 °C to remove any DNA contaminants. The samples were placed at room temperature for 5 min after the addition of the DNase inactivation reagent (0.1 volume). The tubes were centrifuged for 5 min to pellet the reagents, and the supernatant containing the treated RNAs was placed in

a new tube. To ensure the absence of RNA degradation, 4  $\mu$ L of RNA aliquots was incubated at 70 °C for 1 min and loaded on a 1.2% agarose gel (TBE 1X) for 45 min at 90 V. The samples with clear separated bands transducing the absence of RNA degradation were kept for further steps. All the centrifugations were performed at 10,000 g.

### **Quantification and quality check**

The DNA and RNA were quantified with a NanoDrop TM 1000 (THERMO SCIENTIFIC). The sample purity was evaluated by calculating two absorbance ratios. The  $A_{260}/A_{280}$  ratio was used to assess protein contamination and the  $A_{260}/A_{230}$  ratio to determine the contamination by phenolic compounds. In a clean sample,  $A_{260}/A_{280}$  and  $A_{260}/A_{230}$  were approximately 1.8 and 2.2, respectively.

## **2) Molecular constructs and cloning**

The DNA sequences of interest for the different cloning operations were obtained by enzymatic digestion of existing vectors, amplification via PCR or synthesis by an external laboratory (EUROFINS MWG). The cloning strategies were realized depending on the existing plasmids in the laboratory and the restriction enzyme sites present on the plasmids. The strategies of cloning and vector analysis were performed with Vector NTI (THERMO FISHER SCIENTIFIC) software. The cloning of the different constructs was realized by ligation of an insert to a backbone vector. The insert was obtained by vector digestion or following a PCR using primers provided with the appropriate restriction site. In the case of an insert obtained by PCR, the PCR product was cloned in a TOPO vector before being used for cloning. Finally, in some cases, the cloning was realized by InFusion<sup>®</sup> reaction.

### **Agarose electrophoresis**

Digestion products were loaded on an agarose gel for the separation of the fragments according to their size. The gel solution was realized with 1 to 2.5% agarose (UltraPure<sup>™</sup> Agarose THERMO FISHER SCIENTIFIC) depending on the fragment size and TBE buffer (QBIAGEN) (90 mM Tris-HCl, pH 8, 90 mM borate and 2 mM EDTA). Then, ethidium bromide (0.07% solution droplet bottle VWR) was added to the preparation after dissolving the agarose solution. The Trackit<sup>™</sup> 1 kb Plus DNA Ladder (INVITROGEN) was used to determine fragment size on the gel. A virtual digestion of the vector realized with the Vector NTI software was performed to check the size of the fragments observed on the gel.

**DNA fragment amplification**

The DNA fragments used for cloning were amplified by PCR with a high-fidelity polymerase enzyme, iProof<sup>TM</sup> (BIO-RAD). iProof<sup>TM</sup> DNA polymerases possess a low misincorporation rate of  $4 \times 10^{-7}$  (52-fold more accurate than a *Taq* polymerase), a proofreading activity to provide faithful replication of the target DNA and a high processivity of 15-30 kb/s. This polymerase generates blunt-end PCR products. A range of 50-200  $\mu\text{g}$  of DNA was used with 25  $\mu\text{L}$  of iProof Master MIX (BIO-RAD) (0.04 U/ $\mu\text{L}$  iProof, 400  $\mu\text{M}$  dNTPs, 3 mM  $\text{MgCl}_2$ ), 10  $\mu\text{M}$  of forward and reverse primers in a final solution of 50  $\mu\text{L}$ . All PCRs were performed in a C1000 touch thermocycler (BIO-RAD). An initial denaturation step was performed at 98 °C for 3 min. Then, 35 cycles with the following steps were performed: denaturation at 98 °C for 30 s, primer annealing on the DNA at a specific temperature depending on the primer sequence (the melting temperature:  $T_m$ ) for 30 s and extension at 72 °C during the time necessary for the amplified fragment elongation. Then, a final extension was performed at 72 °C for 10 min. To evaluate the specific amplification of a PCR product, 5  $\mu\text{L}$  was loaded on an agarose gel. All the primers used for DNA fragment amplification are listed in Table 5.

Table 5: Primers used for DNA sequence amplification

Primer pair	Primer name	Primer sequence	Primers used for :
Pair 1	BamHI_barstar_F	AGGATCCCAGCTGATGAAAAAGCCGTTATTAACG	Barstar amplification
	3'nos_Xmalb_R	TCCCGGGCTCGAGCGATCTAGTAACATAGATGACACC	
Pair 2	barstarKpnI_F	GAGCCGACGTTACGGTACCCGGTCCAATCTGCAGCCGTC	Constitutive expression of the barstar in bacteria
	barstarKpnI_R	TGCTTGGTTCGTTCCGGTACCGCCGCATACACTATCTCAG	
Pair 3	p35S2_MluI_inf_F	ACAGGGTAATACGCGTAACATGGTGGAGCACGACACTC	p35S amplification
	p35S2_MluI_inf_R	TCTGAATTCAACGCGTTCCTCTCCAAATGAAATGAACTTCC	
Pair 4	p35S2_MluI_inf_F	ACAGGGTAATACGCGTAACATGGTGGAGCACGACACTC	Addition of a TEV sequence after the p35S
	Tev_MluI_inf_R	TCTGAATTCAACGCGTCTATCGTTTCGTAATGGTGA	
Pair 5	pBay347_infAat2_F	ATGTTACTAGATCGGACGTCCAGGGTGTGGAAGTAGGACA	Insulator amplification
	pBay347_infAat2_R	TCACAACCTTGCCGTGACGTCAAAGCCATGATTCAAGTGTGC	

**DNA fragment purification**

For gel fragment purification, 3 volumes of buffer QG were added to 1 volume of harvested gel (100 mg = 100  $\mu\text{L}$ ). The sample was incubated for 10 min at 65 °C to dissolve the gel fragment in the buffer before adding 1 volume of isopropanol. For PCR product purification, 5 volumes of PB buffer were added to 1 volume of PCR. In both purification protocols, the added buffers provided the correct salt concentration for absorption of the DNA to the QIAquick membrane. In the two kits, the solution with the QG or PB buffer was then centrifuged 1 min after being transferred on a QIAquick spin column adapted to the isolation of DNA from aqueous solutions

or agarose gels. The membrane was washed with 750  $\mu\text{L}$  of PE buffer, and a centrifugation step of 1 min to remove any contaminants was performed. The flow-through was discarded, and the empty tube was centrifuged for 2 min. The DNA was eluted with 30  $\mu\text{L}$  of elution buffer (10 mM Tris-HCl, pH 8.5).

### **DNA fragment ligation**

Ligation of two DNA fragments with cohesive or blunt ends was realized by using the viral T4 DNA ligase from the phage T4. The Fast-Link™ DNA Ligation kit (LUCIGEN) was used. The ligation was realized in a final solution of 15  $\mu\text{L}$  with 1.5  $\mu\text{L}$  of the Fast-Link ligation buffer (10 X) (330 mM Tris-acetate [pH 7.5], 660 mM potassium acetate, 100 mM magnesium acetate), 1.5  $\mu\text{L}$  of ATP (10 mM) for ligation of cohesive ends and 0.5  $\mu\text{L}$  for blunt ends, 1  $\mu\text{L}$  of DNA ligase (2 U/ $\mu\text{L}$ ) and the appropriate quantity of DNA insert and vector. For the ligation of cohesive ends, the molar ratio of insert:vector was 2:1, and for the ligation of blunt ends, the insert:vector ratio was set at 5:1. The reaction was incubated for 45 min at room temperature for the ligation and 15 min at 70 °C for ligase inactivation. Six microlitres of the reaction was used for *E. coli* transformation.

### **TOPO cloning**

TOPO cloning of PCR product was realized with a Zero Blunt® TOPO® PCR cloning kit (THERMO FISHER SCIENTIFIC). A mix with 1  $\mu\text{L}$  of the PCR product, 1  $\mu\text{L}$  of salt solution, 1  $\mu\text{L}$  of PCR™ II-Blunt-TOPO® and 2  $\mu\text{L}$  of H<sub>2</sub>O was prepared, and the reaction was performed for 5 min at room temperature. Two microlitres of the reaction was used for *E. coli* transformation.

### **In-Fusion® HD cloning**

In-Fusion® reaction allows fast directional cloning of one or more fragments of DNA into any vector. This process allows homologous recombination between the end sequences of a PCR product and the end sequence of a linearized vector. There must be 15 bp overlap on the flanking sequence of the PCR product and the linearized vector (Figure 31 and Figure 32) (Throop and LaBaer, 2015). This 15 bp overlap can be engineered by designing primers for amplification of the desired sequence (Figure 32). The In-Fusion® HD Cloning (CLONTECH) kit was used, and the cloning reaction was realized as illustrated in Figure 31-b. The solution was incubated 15 min at 50 °C before transfer on ice. Three microlitres of the final reaction was used for *E. coli* transformation.

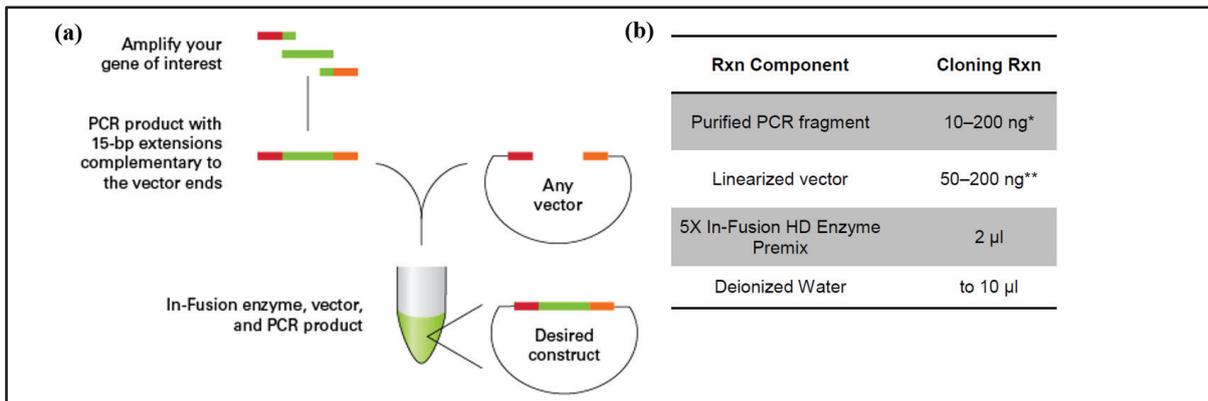


Figure 31: **In-Fusion cloning.** (a) Principle of In-Fusion cloning. (b) Reaction for In-Fusion cloning \* <0.5 kb: 10-50 ng, 0.5 to 10 kb: 50-100 ng, >10 kb: 50-200 ng. \*\*<10 kb: 50-100 ng, >10 kb: 50-200 ng. Rxn: reaction. From the In-Fusion® HD Cloning Kit user manual (CLONTECH).

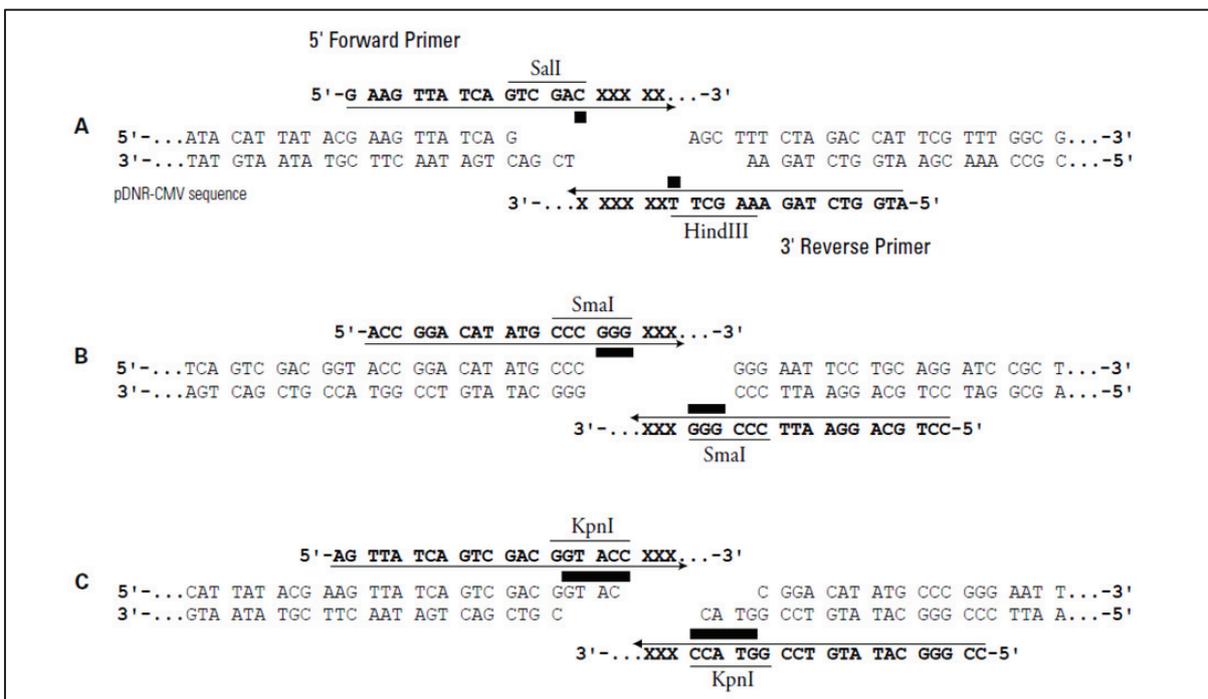


Figure 32: **Examples of primers designed for In-Fusion cloning.** The figure shows examples of primers designed with recognition sites for restriction enzymes that generate 5' overhangs (**Panel A**), blunt ends (**Panel B**), and 3' overhangs (**Panel C**). The primer sequences are shown in bold. X: bases corresponding to the sequence of interest. Additional nucleotides (indicated with a black box) were added to each primer in order to reconstruct the restriction sites. These nucleotides are not part of the 15 bases of sequence homology. From the In-Fusion® HD Cloning Kit user manual.

### 3) Transformation of bacteria

#### Transformation of *Escherichia coli* by thermic choc

One hundred microlitres of thermo-competent bacteria (DH5α<sup>TM</sup> Competent Cells or Stellar<sup>TM</sup> strain) and 6 µL of ligation product or 3 µL of In-Fusion reaction were placed on ice for 15 min to allow DNA binding to the bacteria-permeable wall. A thermic choc for penetration of the

DNA inside the bacteria was conducted by transferring the solution at 42 °C for 45 s and on ice again for 5 min. For transformation of a TOPO vector, 50 µL of DH5α<sup>TM</sup> Competent Cells and 2 µL of the vector were used following the same protocol. Then, 900 µL of SOC liquid medium was added followed by incubation at 37 °C for 1 h at 180 rpm. This step allowed the recovery of bacteria and the expression of the selectable marker. The solution was spread on Lb medium plates supplemented with appropriate antibiotics. The plates were incubated at 37 °C for 24 h to allow transformed bacteria to grow.

### **Transformation of *Agrobacterium tumefaciens* by electroporation**

Twenty microlitres of electro-competent ElectroMAX<sup>TM</sup> *A. tumefaciens* LBA4404 Cells (INVITROGEN), 80 µL of glycerol 10% and 150 ng of vector were placed in a 1 mm cuvette. An electric choc (2k V, 335 Ω, 15 µF for 5 ms) allowing penetration of DNA inside the bacteria was applied with the Equibio Easyject Optima (BIO-RAD) device. A total of 900 µL of liquid Lb was added, and incubation at 27 °C and 180 rpm was performed for 2 h to allow bacteria to recover and the expression of the selectable marker. The solution was spread on an Lb medium plate containing the appropriate antibiotics. The plate was incubated at 28 °C for two days to allow the development of the transformed bacteria.

## **4) Verification of constructs and storage**

### **Screening of bacterial clones**

The analysis of *E. coli* clones was performed either by digestion of extracted plasmid or by PCR on bacterial colonies. *Agrobacterium tumefaciens* colony validation was achieved by PCR on two bacterial colonies per transformed construct.

For the analysis via PCR, a *Taq* polymerase (non-proofreading activity) was used. For one reaction (1 reaction by colony), 10 µM of forward and reverse primers, 10 µL of REDTaq<sup>®</sup> ReadyMix<sup>TM</sup> PCR Reaction Mix (SIGMA-ALDRICH) (20 mM Tris-HCl, pH 8.3, with 100 mM KCl, 3 mM MgCl<sub>2</sub>, 0.002% gelatin, 0.4 mM dNTP, stabilizers, and 0.06 U/mL of *Taq* DNA Polymerase) and 8 µL of water were mixed in a PCR tube. Colony DNA was added by touching with a cone a colony from an Lb plate and soaking the cone in the solution. The PCR was performed in the same conditions as described previously. For a colony screen realized by digestion, plasmid DNA extractions were performed with a 2 mL overnight bacterial culture of the colonies tested. Total PCR or digestion product was transferred to an agarose gel, and one positive clone was selected. Then, plasmid DNA of the validated clone was used to perform digestions at different sites all around the construct. Three mixtures of digestions were created

with different restriction enzymes. If the results were positive, the plasmid was sequenced by EUROFINs from the left border (LB) to the right border (RB). The obtained sequences were aligned with the vector map using Vector NTI software.

### **Bacteria storage**

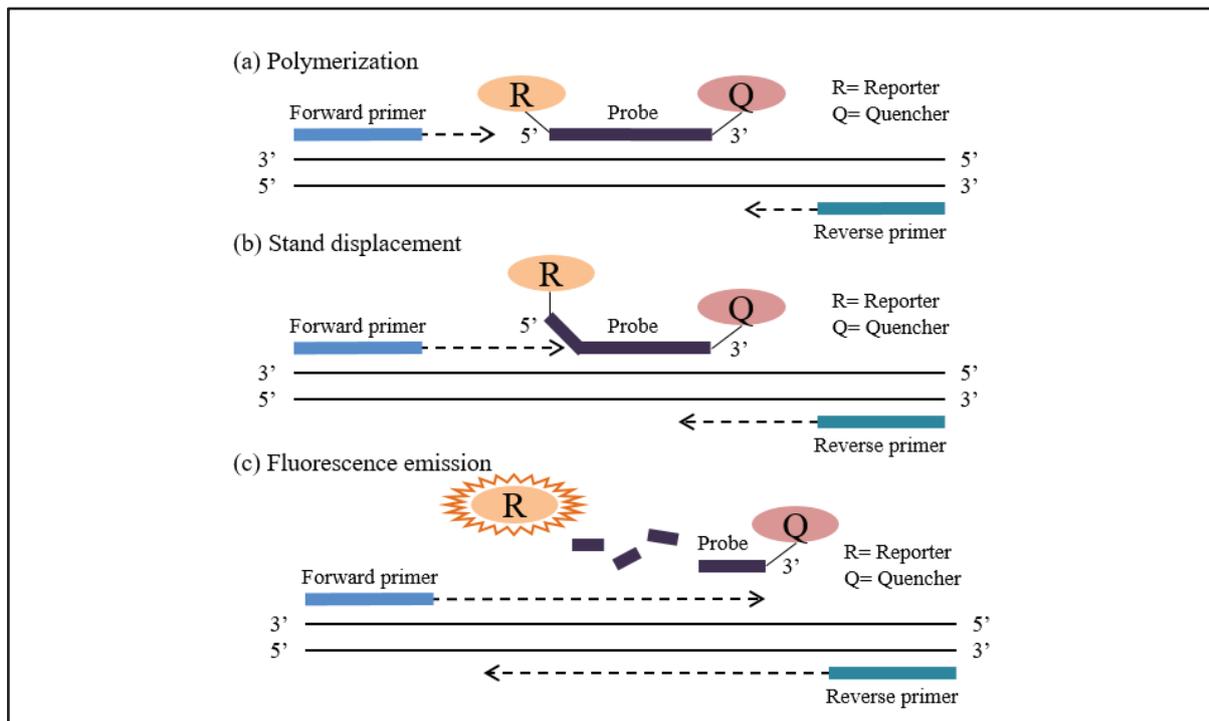
The created plasmids were entered in the Bayer Vectoring Platform database, and a glycerol stock was created with 1.2 mL of bacterial culture and 0.6 mL of sterile glycerol 50%. The stock was kept at -80 °C. All vectors used in this study are listed in Table 6.

Table 6: Vectors used in the study

Plasmid name	Cassette of interest	Use for:
pBay00457	p <i>GmCHIT1</i> :GFP	Candidate promoter evaluation
pBay01065	p <i>GmRIM</i> :GFP	
pBay01108	p <i>gst1</i> :GFP	
pBay02613	p <i>GmASP</i> :GFP	
pBay01351	pNOS:barstar	Evaluation of the cell death system
pBay01404	p <i>gst1</i> :barnase p35S:barstar	
pBay01405	p <i>gst1</i> :barnase pNOS:barstar	
pBay01834	p <i>GmCHIT1</i> :barnase p35S:barstar	
pBay01842	p <i>GmCHIT1</i> :barnase pNOS:barstar	
pBay02475	p <i>GmCHIT1</i> :barnase insulator p35STEV:barstar	

### **5) Analysis of the number of inserts in transgenic plants by ddPCR**

The analysis of insert number in transgenic plants was realized via digital droplet PCR (ddPCR). This technique measures the DNA quantity of a sample by counting nucleic acid molecules encapsulated in a water-in-oil droplet partition. First, a real-time PCR was performed with FAM and HEX reporter fluorophores probes (BIO-RAD) acting similar to TaqMan hydrolysis probes (Figure 33).



**Figure 33: TaqMan probe principle** A TaqMan Probe is an oligonucleotide labelled with two different dyes. A reporter dye (R) is located at the 5' end, and a quencher molecule (Q) is located at the 3' end. The quencher molecule inhibits the natural fluorescence emission of the reporter. (a) The primer is elongated by the polymerase, and the probe binds to the specific DNA template. Hydrolysis releases the reporter from the probe/target hybrid (b), causing an increase in fluorescence (c). The measured fluorescence signal is directly proportional to the amount of target DNA. In our case, two probes were used, one with FAM and the other with HEX Reporter dyes. From THERMO FISHER SCIENTIFIC.

The natural emission of the reporter (FAM or HEX fluorophore) is inhibited by a quencher. When the reporter is released by amplification of the targeted DNA, an increase in fluorescence is observed. For one reaction, 10 ng of genomic DNA, 1.05  $\mu\text{L}$  of *Lectin* primer and *HPPD-4* primers, 0.52  $\mu\text{L}$  of *Lectin* probe, 0.21  $\mu\text{L}$  of *HPPD-4* probe, 1  $\mu\text{L}$  of EcoRI, 10.5  $\mu\text{L}$  of ddPCR<sup>TM</sup> Supermix for Probe (no dUTP) (BIO-RAD) and 18.6  $\mu\text{L}$  of water were used. Twenty microlitres of the reaction and 70  $\mu\text{L}$  of Droplet Generator Oil were mixed in the appropriate DG8<sup>TM</sup> cartridge wells, and the cartridge was placed in the QX100 Droplet Generator (BIO-RAD). This device will partition each sample in 20,000 droplets. The target and the background DNA were distributed randomly into the droplets (Figure 34-a). The droplets were transferred to a 96-well plate. The plate was sealed (180 °C for 2 s) and placed in the C1000 Touch<sup>TM</sup> Thermal Cycler (BIO-RAD) for PCR. Following the PCR amplification of the nucleic acid targeted in the droplets (Figure 34-b), the plate was placed in a QX100 Droplet Reader 5 (BIO-RAD). The apparatus will analyse each droplet individually using a two-colour detection system set to detect FAM and HEX fluorescence (Figure 34-c). Each reaction will provide a fluorescent positive or negative signal indicating whether the target DNA is present or absent. The fraction of positive droplets was used to calculate the target DNA concentration.

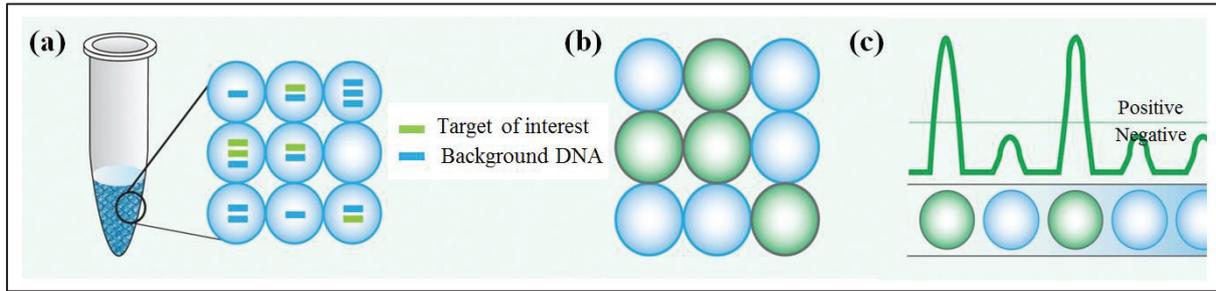


Figure 34: **Digital droplet PCR principle** (a) For each sample, the PCR mixture is partitioned into 20,000 water-in-oil droplets with targeted and background DNA randomly distributed among the reaction. (b) Target DNA is amplified by PCR (droplet in green). (c) The fluorescence intensity is measured in each droplet. From BIO-RAD.

This technique can be used to follow the DNA quantity of multiple target DNA sequences. In our case, it was used to determine the variation in the number of inserts according to an invariant reference gene. As our selection marker of transgenic soybean is HPPD, specific primers of the *HPPD-4* gene transferred in transgenic plants and a FAM probe of the *HPPD-4* gene were used to look for the insert number in the transgenic soybeans (Table 7). The lectin gene *Glyma.02G012600*, present twice in the soybean genome (Schmutz *et al.*, 2010), was used as a reference. Specific primers and a HEX probe for the reference gene are listed in Table 7. The number of *HDDP-4* insertions was determined by calculating the ratio of targeted molecule (*HPPD*) concentration to the reference molecule (*Lectin*) concentration times the number of copies in the genome (2 in the case of the soybean).

Table 7: **Probes and primers used in ddPCR**

Gene	Probe / Primers	Name	Sequence
<i>HPPD-4</i>	Probe	Hpw336-1Pd_S	FAM-CGTGTTGGATTCCCTCACCGTTAAACTG-BHQ1
	Primers	PS_hpw336-1Pd_F	CAAGATCGTCTGTCAAAAAAGCA
		PS_hpw336-1Pd_R	GCTGGACAAATCGAAGAGTTCCT
<i>Lectin</i>	Probe	GmLec_S	YakYe-CCACAAACACATGCAGGTTATCTTGG-BHQ1
	Primers	GmLec_F	CTTCTCGCACCAATTGACA
		GmLec_R	TCAAACCAACAGCGACGAC

## 6) Reverse transcription (RT)

A ThermoScript™ RT-PCR System kit (INVITROGEN) was used for the reverse transcription of the RNA. For one sample, 1 µL of oligo dT (50 µM), 2 µL of dNTP Mix (10 mM), 1 µg of RNA and H<sub>2</sub>O were used in a final solution of 12 µL. The solution was incubated at 65 °C for 5 min for denaturation of the RNA. Four microlitres of cDNA synthesis buffer (5 x), 1 µL of DTT (0.1 M), 1 µL of RNase OUT (40 U/ µL), 1 µL of H<sub>2</sub>O and 1 µL of ThermoScript RT (15 U/µL) were mixed with the sample and incubated 1 h at 50 °C and 5 min at 85 °C for cDNA synthesis. The remaining RNA was degrading by adding 1 µL of RNase H to the sample before incubation at 37 °C for 20 min.

## 7) Quantitative PCR (qPCR)

Real-time quantitative PCR allows the measurement of products generated during each cycle of the PCR. For amplicon detection, SYB Green (BIO-RAD) was used. When intercalated into double-strand DNA, the SYB Green fluoresces, and this fluorescence is measured at each PCR cycle. When the signal recorded is above the minimal detection level (i.e., the threshold), the number of threshold cycles (Ct) is recorded. To perform relative qPCR, the comparative threshold method was applied (Arya *et al.*, 2005).

The cDNA was diluted at 0.02 µg with RNase-free water to a final volume of 100 µL. Five microlitres of diluted cDNA (0.1 µg of cDNA in total) was used in a 20 µL reaction containing 10 µL of SsoAdvanced™ Universal SYBR® Green Supermix (BIO-RAD), 6 µM of primer forward and reverse and 3 µL of RNase free water. The qPCR was performed using the LightCycler® 480 (ROCHE). The expression of soybean genes was followed by using specific primers (Table 8). The thermocycling conditions were as follows: initial denaturation at 95 °C for 10 min followed by 45 cycles of 10 s at 95 °C (denaturation), 10 s at 60 °C (primer annealing) and 10 s at 70 °C (elongation). After the final cycle, the dissociation curve analysis was carried out to verify that the amplification occurred specifically and that no primer dimer product was generated. The actin and hypothetical protein genes (Hirschburger *et al.*, 2015) (primer sequence in Table 8) were used as endogenous reference genes to normalize the calculation using the comparative Ct value method. The level of transcript abundance relative to the reference gene (termed  $\Delta Ct$ ) was determined according to the function  $\Delta Ct = Ct$  (test gene) -  $Ct$  (reference gene). Then, the  $\Delta \Delta Ct$  was first determined using the equation  $\Delta \Delta Ct = \Delta Ct$  (treatment) -  $\Delta Ct$  (control) (where control represents mock-treated plants). The ratio of

treatment/control was calculated by the equation  $2^{-\Delta\Delta Ct}$ . All the calculations were realized with LightCycler® 480 SW 1.5 software (ROCHE).

Table 8: **Primer sequences for RT-qPCR.**

Gene	Primer name	Primer sequence	Amplicon length
Actin	GmACTIN_F	CGGTGGTTCTATCTTGGCATC	142 bp
	GmACTIN_R	GTCTTTCGCTTCAATAACCCTA	
Hypothetical protein	GmUKN2_F*	GCCTGGATACCTGCTCAAG	79 bp
	GmUKN2_R*	ACCTCCTCCTCAAACCTCCTCTG	
<i>GmRIM</i>	GmRIM_F	GTACTTGGGCTTAATAAGAGGGTTA	76 bp
	GmRIM_R	GCCCATTTTCACCATAGACTTC	
<i>GmEXPLB</i>	GmEXPLB_F	GGCTTTGGTGAATATGGAAGAG	82 bp
	GmEXPLB_R	CAGCTCCATTCTCCATAGAT	
<i>GmCHIT1</i>	GmCHIT1_F	GAGATTAACGGTGCATCAGG	330bp
	GmCHIT1_R	ATTAACACGAGCCTGAACAGTACT	
<i>GmPR1</i>	GmPR1_F**	AACTATGCTCCCCCTGGCAACTATATTG	76bp
	GmPR1_R**	TCTGAAGTGGTAGCTTCTACATCGAAACAA	
<i>GmPR2</i>	GmPR2_F ***	GTCTCCTTCGGTGGIAGTG	104bp
	GmPR2_R ***	ACCCTCCTCCTGCTTTTCTC	
<i>GmPR3</i>	GmPR3_F ***	GCATGGTCTGGATTG	115bp
	GmPR3_R ***	GGCTTGATGGCTTGTTTC	
Elongation factor	GmELF_F	GTTGAAAAGCCAGGGGACA	79bp
	GmELF_R	TCTTACCCCTTGAGCGTGG	

Each primer pair possesses an efficacy of 2. \*from Hirschburger *et al.*, 2015, \*\* from Mazarei *et al.*, 2007, \*\*\* from Zhong *et al.*, 2014.

## 8) Analysis of the pBay01404 events

A PCR on the baranse, barstar, HPPD sequences and a sequence outside the LB/RB borders of the pBay01404 plasmid was realized (primer in Table 9). For one reaction 10  $\mu$ M of forward and revers primers, 10  $\mu$ L of REDTaq® ReadyMix™ PCR Reaction Mix (SIGMA-ALDRICH) (20 mM Tris-HCl, pH 8.3, with 100 mM KCl, 3 mM MgCl<sub>2</sub>, 0.002 % gelatin, 0.4 mM dNTP, stabilizers, and 0.06 U/mL of *Taq* DNA polymerase) and 8  $\mu$ L of water were mixed in a PCR tube. Plant DNA (1  $\mu$ L) was added to the reaction. The PCR was realized in the same conditions as described previously.

Table 9: Primer used for the analyse of the pBay01404 T0 events

Primer pair	Primer name	Primer sequence	Primers used for :
Pair 1	Barstar_F	CCTCTAGACCCAAGCTTGCG	Barstar amplification
	Barstar_R	ACAGTGGTCCCAAAGATGGA	
Pair 2	Barnase_F	GGGAGTCAAATTGACCGATCAGAG	Barnase amplification
	Barnase_R	GCTATGGTACCGGTTATCAACACG	
Pair 3	pTCv17_kpn1_F	ACCGGTACCCAAAAAAGCGGTTAGCTC	Amplification of a sequence outside the LB/RB borders of pBay01404
	pTCv17_kpn1_R	ACCGGTACCGTATTATCCCGTGTGAC	
Pair 4	Hppd-F	AGCCGTCATCTCCTTTACGC	HPPD amplification
	Hppd_R	TCAATCAGCAGTAAGAACACC	

## C. BIOCHEMISTRY

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### 1) Extraction and quantification of plant proteins

Four foliar discs of soybean were punched per sample for protein extraction. A total of 250  $\mu\text{L}$  of extraction buffer (Tris-HCl 100 mM, NaCl 100 mM and DDT 0.04%) was added to each sample. The tubes were vortexed for 3 min, transferred to ice for 10 min and centrifuged at 4  $^{\circ}\text{C}$  for 10 min. A total of 150  $\mu\text{L}$  of the supernatant was kept, and the protein concentration was determined with the Bradford method (Kruger, 1994) using the BIO-RAD Protein assay dye reagent solution. The protein optical density (OD) was measured at 595 nm with the NanoDrop<sup>TM</sup> 1000 (THERMO SCIENTIFIC). Then, the following formula was applied to calculate the sample concentration:

$$[(\text{OD}_{595 \text{ nm}} - 0.0074) / 28.022] \times \text{dilution ratio} = \text{protein concentration } (\mu\text{g} / \mu\text{L})$$

### 2) Western blotting

For protein denaturation, 1 volume of Laemmli buffer (2 X) (BIO-RAD) was added to 1 volume of extracted proteins (30  $\mu\text{g}$ ). The solution was maintained 5 min at 95  $^{\circ}\text{C}$  and 5 min on ice before being loaded on a TGX 4-20% Strainfree (BIO-RAD) gel with the TGS 1X (BIO-RAD) buffer. After migration, the gel was transferred on a membrane by using the kit Trans-Blot<sup>®</sup> Turbo<sup>TM</sup> Midi Nitrocellulose Transfer Packs (BIO-RAD) and the TransBlot Turbo device (BIO-RAD).

Then, the membrane was transferred to 30 mL of blocker solution (Western Blocker for HRP detection system, SIGMA) under slow agitation to avoid unspecific binding of the first antibody. After 1 h the solution was replaced by the first antibody (for either GFP, barnase or barstar detection) diluted at 1/4000 in the blocker solution. After 1 h under slow agitation, the membrane was washed 3 times for 10 min with 50 mL of TTBS 1X solution (100 mL of TBS 10X BIO-RAD, 500  $\mu\text{L}$  of Tween 20 and 900 mL of water).

The membrane was then transferred to a solution with the Immun-Star Goat Anti-Rabbit (GAR)-HRP Conjugate antibody (BIO-RAD) diluted at 1/25 000 in TTBS 1X for 1 h under slow agitation. This second antibody is labelled with the HRP enzyme that allows the detection

of the first antibody with the appropriate substrate. An additional washing was performed 3 times for 10 min with 50 mL of TTBS 1X solution and once for 5 min with 50 mL of TBS 1X. The results were determined with the Clarity™ Western ECL (BIO-RAD) kit by following the supplier's instructions. Finally, the ChemiDoc™ Touch camera (BIO-RAD) was used for detection of the results by chemiluminescence.

## D. CELLULAR BIOLOGY

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### 1) Stable transformation of soybean

Stable transformation of soybean was realized by *Agrobacterium tumefaciens* LBA4404 strain using the cot-nod technology developed in the laboratory, patented under WO 2011/095640 but not published. All the tissue culture steps from cotyledon transformation to shoot development were realized in a sterile environment. The environmental conditions for the tissue culture were the following: temperature 24 °C, 16 h light (180  $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ), 8 h dark and 75% relative humidity.

#### Cot-nod transformation

Seeds were surface sterilized for 24 h by chlorine gas generated with a mixture of 150 mL Domestos containing 4.5% NaClO w/w (Unilever) and 5 mL of HCl (37%). Sterile seeds were then hydrated overnight in sterile deionized water (Figure 35-a). Cotyledons of germinated seeds were dissected to ensure contact between the bacteria and the vegetal cells. The seeds were split into two halves using a scalloped blade in order to remove the seed coat and the embryo before being sonicated for 2 min to create micro-injuries (Figure 35-b). The half-seeds were immersed for 30 min in a culture of *A. tumefaciens* bacterium (final OD<sub>600nm</sub>: 0.8) in a co-culture medium (CCM). The CCM medium was composed of 10% W/V Gamborg's medium (Gamborg *et al.*, 1968) containing major and minor salts and vitamins, 30 g/L sucrose, 7.4  $\mu\text{M}$  BAP, 0.7  $\mu\text{M}$  GA3 for growth and elongation, 3.3 mM cysteine for shoot induction, 1 mM dithiothreitol, 200  $\mu\text{M}$  acetosyringone for the activation of *vir* genes and 20 mM MES at pH 5.4. Cotyledons were then transferred to 12.5 mm Petri dishes, adaxial side down, onto 3 layers of Whatman® paper pre-soaked with 10 mL of CCM medium (Figure 35-c). The plates were transferred in a tissue culture room for 5 days to allow the process of transformation.

#### Shoot development

Shoots were induced by transferring the cotyledons in the shoot induction (SI) medium containing full-strength Gamborg's medium with 30 g/L sucrose, 7.4  $\mu\text{M}$  BAP, 3 mM MES pH 5.6 and 8 g/L noble agar. To prevent shoot contamination by bacteria, antibiotics (ticarcillin 50 mg/L, cefotaxime 50 mg/L, vancomycin 50 mg/L) were added to the SI medium after autoclaving as well as the herbicide Tembotrione™ (TBT) (0.2 mg/L) used as a selectable

marker (Figure 35-d). After one month on the SI medium, non-transformed white shoots (TBT sensitive) were removed and cotyledons were transferred to a shoot elongation (SE) medium containing Murashige & Skoog (Murashige & Skoog, 1962) salts, Gamborg's vitamins, 30 g/L sucrose, 100 mg/L pyroglutamic acid, 50 mg/L asparagine, 0.28  $\mu\text{M}$  zeatin riboside, 0.57  $\mu\text{M}$  indol-3-acetic acid for shoot elongation, 14.8  $\mu\text{M}$  GA3 for growth and elongation, 3 mM MES pH 5.6 and 8 g/L agar (Figure 35-e). Antibiotics and the herbicide were still added at the same concentration. After one month on this medium, elongated shoots were cut and transferred to a rooting medium (Figure 35-f) consisting of half-strength MS salts, half-strength B5 vitamins, 15 g/L sucrose, 8 g/L agar, and no pH adjustment. The same antibiotics were added after autoclaving, but the selectable marker was omitted.

### Transfer of rooted shoots to the greenhouse

When roots were sufficiently developed, the shoots were removed from agar and transferred to 7x7 cm pots containing SteckMedium substrate for 10 to 15 days. For better efficiency of recovery, the pots were placed in an aerated plastic bag and transferred to a mini greenhouse inside the large greenhouse. Well-developed shoots were then removed from the plastic bag but kept for two additional days in the mini greenhouse before being cultivated (Figure 35-g) using the conditions described in the plant material section.

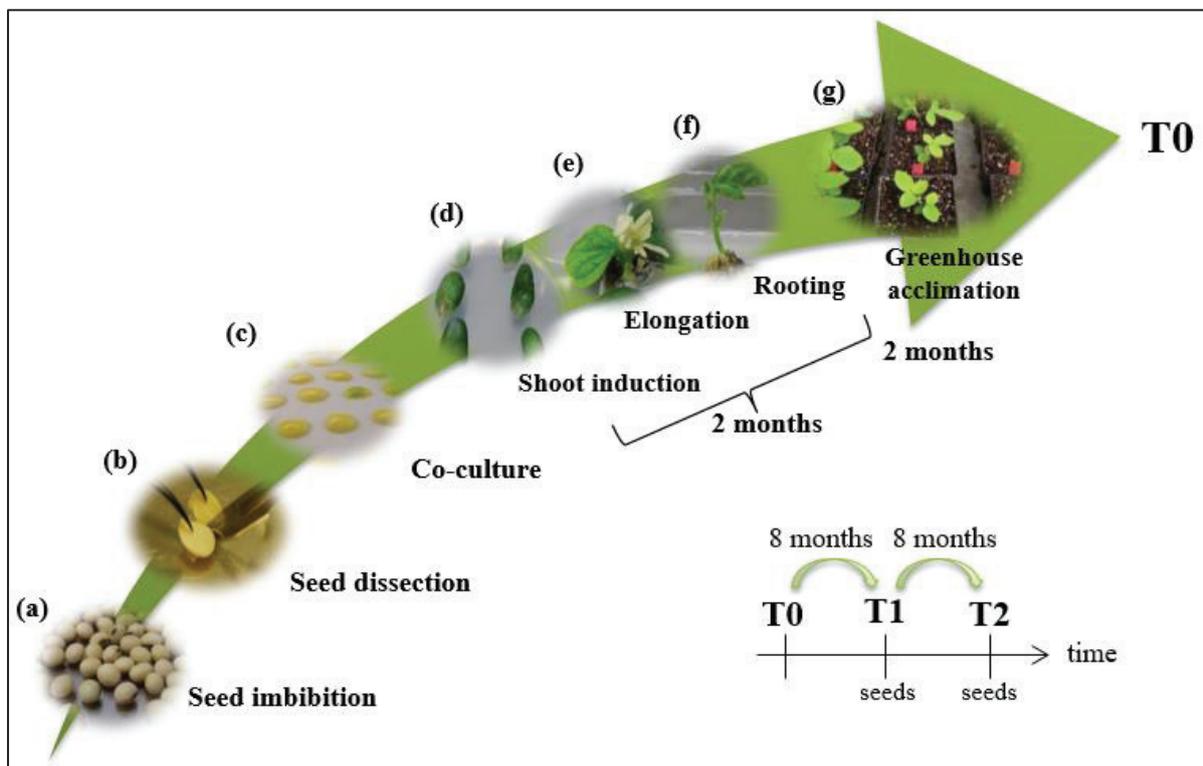


Figure 35: Stable transformation of soybean by *Agrobacterium tumefaciens*. (a) Imbibition of sterile seeds. (b) Dissection and preparation of cotyledons. (c) Co-culture of the cotyledons with *A. tumefaciens*

containing the construct of interest. (d) Cotyledons were placed on the shoot induction medium in the presence of the selectable marker: TBT. (e-f) Shoot elongation. Negative shoots were white, and those containing the HPPD gene were green. Only green shoots were transferred to the rooting medium and later to the greenhouse (g).

## 2) Characterization of transgenic soybean plants

Regenerated T0 events were confirmed for the presence of the selectable marker gene with an HPPD lateral flow test (AMAR Immunodiagnostics) using the experimental instructions recommended by the provider. To detect T1 HPPD-4 positive events, germinated seeds were watered with an 8 ‰ solution of the herbicide Isoxaflutole™ to eliminate null segregant plants. Plants showing no herbicide symptoms were used for further analysis. Homozygous single-locus plants were preferentially selected either in T1 or T2 segregating generations by ddPCR analysis. T0, T1 or T2 plants were used depending on the availability of the material.

## 3) Transient transformation of *Glycine max*

A 2-day culture of *A. tumefaciens* strain EHA105 with the appropriate construct was centrifuged and re-suspended in 55 mL of suspension buffer (20 g/L sucrose, 10 mM MES-KOH pH 5.7 and 200 µM of acetosyringone). Bacteria were incubated at room temperature for 1 to 3 h to allow the activation of *vir* genes. The bacterium suspension was adjusted at  $OD_{600} = 0.9$  and infiltrated with a vacuum (two times 1 bar for 45 s) into the first leaves of 3-week-old *G. max* plants.

## 4) Transient transformation of *Nicotiana benthamiana*

A 3 day-culture of *A. tumefaciens* strain LBA4404 with the appropriate construct was centrifuged and re-suspended in 15 mL of suspension buffer (10 mM MgCl<sub>2</sub>, 10 mM MES-KOH pH 5.7 and 150 µM of acetosyringone). Bacteria were incubated at room temperature for 3 to 4 h to allow the activation of *vir* genes. The bacterium suspension was adjusted to  $OD_{600} = 0.4$  and infiltrated with a needleless syringe (10 mL) into the abaxial surface of *N. benthamiana* leaves from 5-week-old plants. The infiltrated area was approximately 1.5 cm in diameter, and 2 or 4 infiltrations were performed on 3 leaves of the same plant. The plants were placed in a mini greenhouse and transferred to a growth chamber used for soybean tissue culture. The observations were performed 4 days post-infiltration.

## 5) Fungal contamination of entire plants of soybean or detached leaves

### Inoculation for RT-qCPR analysis and GFP fluorescence observation

After rehydration (one night at 21°C in the dark), the spores of *P. pachyrhizi* were suspended for 30 min in sterilized water containing 0.01% Tween 20 to reach a final concentration of 100,000 spores/mL. For the contamination of entire plants (WT or transgenic plants generation T1), 3-week-old soybeans were used and sprayed until run-off. The inoculated plants were incubated in a growth chamber (temperature 24 °C, dark, 100% relative humidity) for 24 h before being transferred to a developing chamber (temperature of 24 °C, 16 h light/8 h dark, light intensity 15  $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  and 80% relative humidity). For contamination of detached leaves (transgenic plants generation T0), leaves were excised and transferred to 12.5 mm Petri dishes containing two layers of Whatman<sup>®</sup> paper (SIGMA ALDRICH) wetted with 6 mL of sterile distilled water. Petioles were wrapped with water-soaked cotton. The detached leaves were sprayed with the spores until run-off and then incubated using the same conditions as used for entire contaminated plants. Samples from detached leaves and entire plants were harvested at different times after contamination for microscopic observations or RT-qPCR analysis.

### Inoculation for disease assessment

The inoculations are realized on T0 detached leaves or T1 entire plants exactly as described previously except that the inoculum concentration was set to 1000 spores/mL. The % of infection assessment on the T1 plants was realized following the diagrammatic scale of soybean rust severity developed and validated by Godoy *et al.* (2006) (Figure 36).

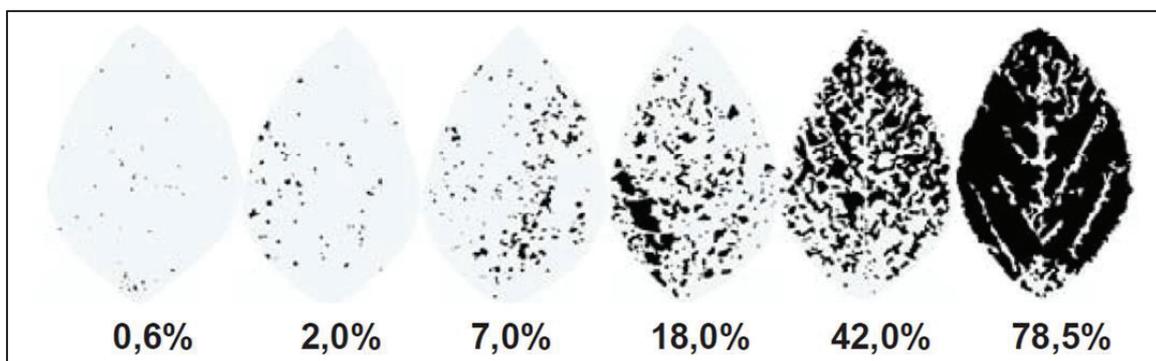


Figure 36: **Diagrammatic scale of soybean rust severity.** Percentage of infection, *i.e.*, leaf area covered by the disease. From Godoy *et al.*, 2006.

## 6) Hormonal and wounding treatments of soybean detached leaves

Leaves of soybean plants were excised and transferred in 12.5 mm Petri dishes containing two layers of Whatman® paper (SIGMA ALDRICH) wetted with 6 mL of sterile distilled water. Leaf petioles were wrapped with water-soaked cotton to increase organ survival. Different hormone treatments were conducted by spraying leaves with either 20 mM of 1-aminocyclopropane-1-carboxylic acid (ACC, ethylene precursor) or 2.5 mM solution of salicylic acid (SA) in sterile water or 3 ppm of coronatine (methyl jasmonate analogue) in 1% EC premix solution (phenyl sulfonate 5%, emulsogen EL360 7%, isophoron 40% and methyloleate 48%). Sterile distilled water was used as mock for ACC and SA treatment, and 1% EC premix was used as mock for the coronatine spray. Leaf wounding was realized with a sterile scalpel blade. After the different treatments, the leaves were incubated in the same growth chamber used for soybean transformation. Macroscopic observations and, in some cases, fluorescence intensity measurement were performed.

## E. MACRO-MICROSCOPY

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### 1) Dissection scope microscopy

GFP fluorescence was analysed with a Leica Z16 APO A dissection scope equipped with a GFP filter. For hormonal treatments and wounding, the following parameters were used: camera lens 1 x, magnification 6.95 x, gain 3 and exposure time 1 s. For the detection of fluorescence after rust inoculation, the parameters were set as follows: camera lens 1 x, magnification 115 x, gain 2 and exposure time 500 ms. For detection of the GFP fluorescence in the different soybean tissues without infection, the camera was set at lens 0.5 x, magnification at 6.95 x for roots and young trifoliolate leaves and 15 x for flowers, gain 3, exposure time 500 ms. Fluorescence intensity measurement was performed with MetaMorph software (MOLECULAR DEVICES) via greyscale value.

### 2) Confocal microscopy

Soybean leaf samples of WT and event 131 expressing the transcriptomic fusion *pGmCHIT:GFP* were harvested 24 h post-*P. pachyrhizi* contamination. The samples were first stained in an aqueous calcofluor white solution (0.01 mg/mL) for 5 min before being washed 3 times in water for 5 min for detection of fungal appressoria and germ tubes. Samples were mounted in water under slides (VWR<sup>®</sup> microscope slides: ground edges 45 °, 76 x 26 mm) and cover glass (VWR<sup>®</sup> cover glass: 22 x 32 mm). Observations were conducted with a ZEISS LSM 800 microscope using the 10x objective. To visualize GFP fluorescence, a 487 nm wavelength laser was used for excitation, and light emission was captured at 560 nm. For the imaging of calcofluor fluorescence, light excitation was set at a wavelength of 400 nm and emission was captured at 487 nm.

## F. BIOINFORMATIC ANALYSES

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For gene expression analysis in response to the soybean rust, internal RNA-seq data generated by Bayer Crop Science were used. Experimental designs were performed by Catherine Sirven, and the experiments were conducted by Marylène Buiron (Bayer employees). RNA sequencing, data mapping and counting were performed by Stéphane Peyrard and Pierrick Gautier (Bayer employees).

### 1) RNA sequencing, mapping and counting

RNA-Seq technology can be used to determine RNA expression levels. Briefly, a population of RNA is converted to a library of cDNA fragments with adaptors attached to their ends. Each molecule is then sequenced. Short sequences called reads (30 to 400 bp depending on the DNA-sequencing technology) are obtained. The resulting reads are aligned to a reference genome to calculate the expression level for each gene (Wang *et al.*, 2009).

RNAs from the different samples (see samples used for RNA sequencing) were sequenced by Fasteris, and raw sequencing data were obtained in FastQ format. Sequencing data quality was assessed by FastQC software developed at the Babraham Institute. Reads were mapped on the soybean genome (*Glycine max* 275 William 82) using Bowtie2 through RSEM package version 1.2.20 (Li and Dewey, 2011). Read counting was also realized with the RSEM package version 1.2, and FPKM (fragments per kilobase per million mapped reads) was calculated. These two previous steps were integrated into a Bpipe pipeline (Sadedin *et al.*, 2012) including an in-house Python script. Last output files were formatted and fused for integration within the appropriate software.

### 2) Gene expression analysis

Counting data from internal RNA-seq experiment (FPKM associated with soybean genes in each sample) were imported into GeneData Analyst software for differential gene expression analysis. Finally, gene annotation was added from Phytozome (<https://phytozome.jgi.doe.gov/pz/portal.html>) into GeneData Analyst.



# CHAPTER 1

## IDENTIFICATION OF CANDIDATE PROMOTERS



## A. INTRODUCTION

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The choice of the appropriate promoter to control gene expression is an essential point in biotechnology approaches. This is particularly true for strategies aiming to induce cell death, such as the one we seek to develop with the barnase/barstar system. This technology is based on a barnase ribonuclease highly toxic for the plant cell and which consequently need to be expressed only in the presence of the pathogen. The lethal expression of the barnase is counterbalanced by barstar a highly specific inhibitor of the barnase. The objective is to achieve is a “proof of concept” to determine if this technology can control the disease. In this system, the most important point is to obtain the right balance of barnase and barstar proteins during the whole cycle of crop development and particularly when the crop is challenged by the rust.

To trigger a local and precise cell death under pathogen attacks only, the barnase expression has to be controlled by a promoter inducible by *P. pachyrhizi*. The selected promoters must have a relatively low expression in non-infected soybean tissues to avoid non-desired cell death and must reach a high expression following a soybean rust infection. We use a constitutive promoter to drive the barstar gene expression in order to counterbalance any possible barnase background without pathogen infection. Moreover, the barnase expression must exceed the barstar expression during the infection (Figure 37). Intuitively, a relatively high induction level would be ideal. Thus, different strengths of promoters might fit with the system to trigger the cell death, giving some flexibility in the choice of promoters. In addition, the barnase promoter should be not induced by biotic or abiotic stimuli except *P. pachyrhizi*. Figure 37 summarizes the necessary kinetics of the barnase/barstar system using the appropriate inducible promoters.

This first chapter is dedicated to the identification of candidate promoters potentially suitable to drive the *barnase* gene expression. The identification of such candidate promoters was carried out using 1) transcriptomic data and 2) data from the literature.

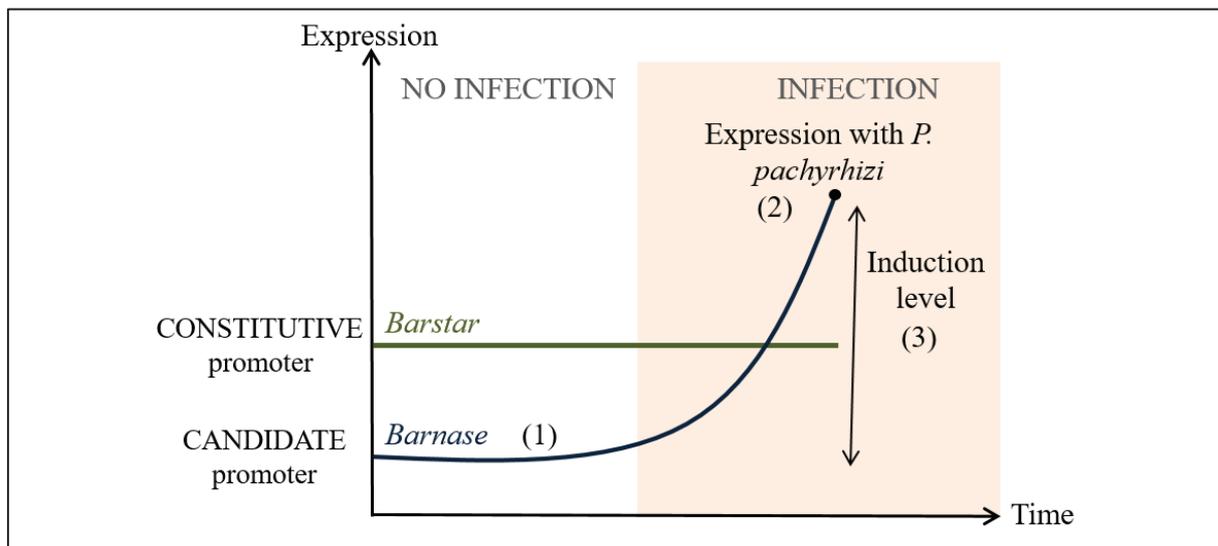


Figure 37: Schematic representation of candidate promoter expression following *P. pachyrhizi* infection. (1) The appropriate candidate promoters must control the expression of the barnase with low basal expression in all tissues. (2) The barnase expression must be induced in infected tissues to exceed barstar expression. (3) Importance of the induction level in infection conditions.

## B. GENE EXPRESSION ANALYSIS

To identify candidate promoters, we used data (from RNA-seq) of soybean (*G. max*) gene expression during the early period of infection by *P. pachyrhizi*. These data have been generated by the host laboratory and were available for this study. RNA from infected and non-infected leaves were collected at different times post-infection—0, 0.5, 1, 2, and 24 hpi (Figure 38-a)—and sequenced. Gene expression data after treatment with chitin oligosaccharides (the chitinheptaose DP7) were also available. RNA from treated (DP7) and non-treated (water) leaves were collected at 0, 0.5, 1, 2, and 24 h after treatment and sequenced (Figure 38-a).

All these data were used to identify candidate genes rapidly induced by the pathogen and not by a DP7 treatment, thus highlighting genes potentially specifically induced by the pathogen. Identifying these genes was a way to obtain pathogen inducible promoters and candidate promoters for the *barnase* expression.

Considering the infection process of *P. pachyrhizi*, spore germination occurs between 1-4 h and germ tubes develop an appressorium structure. At 24 h, the pathogen grows through the mesophyll cells to form haustorium structures (Figure 38-b).

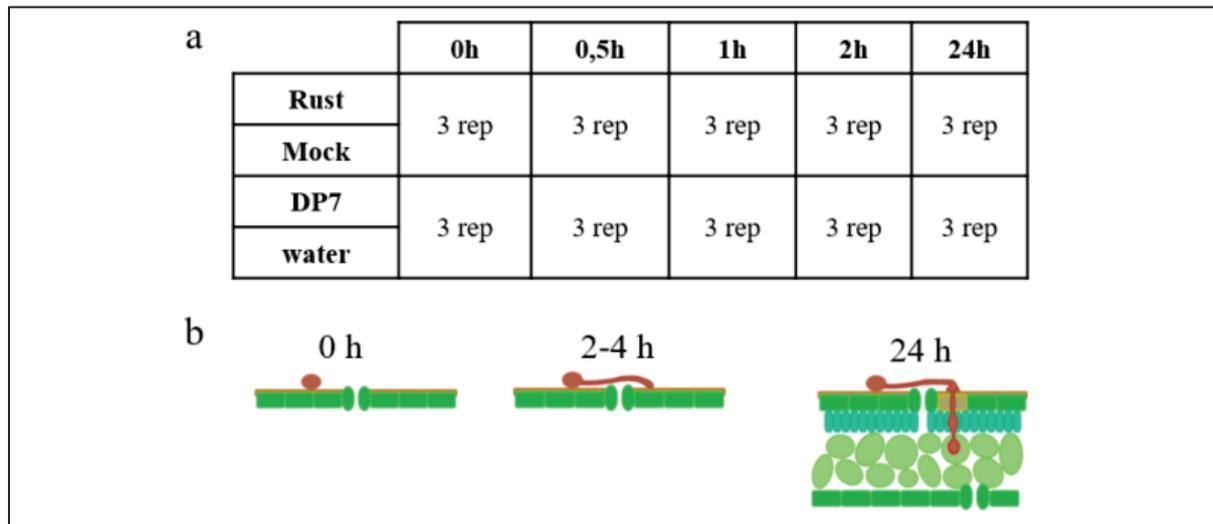


Figure 38: **Samples used to generate transcriptomic data during soybean rust contamination and after a DP7 treatment.** (a) RNA of infected (rust), non-infected (mock), treated (DP7) and non-treated (water) soybean were harvested at 0, 0.5, 1, 2, and 24 h and used for sequencing. In each condition, 3 biological replicates (rep) were included. (b) Representative schema of the fungal development at different time points.

The mapping of the sequencing results on the soybean genome, the calculation of the gene expression in FPKM (fragments per kilobase per million of mapped reads) and the quality checks of the data were performed by S. Peyrard and P. Gauthier (both Bayer CropScience). The “GeneData Analyst” software (<https://www.genedata.com/>) was used for gene expression analysis.

### 1) Candidate gene selection

The RNA-seq data generated with infected and non-infected soybean leaves were used. For each time point, all the expressed genes after mock inoculation and *P. pachyrhizi* infection were compared. Only the deregulated genes exhibiting an expression significantly different (*t. test*:  $p$  value  $< 0.05$ ) between the mock and the infected tissues in the three replicates were further studied. The relative expression  $\left( \frac{\text{expression with } P.pachyrhizi \text{ (FPKM)}}{\text{expression in mock (FPKM)}} \right)$  of all the deregulated genes was determined, and the relative expression mean of the three replicates was calculated. The deregulated genes were filtered, and only those with a mean relative expression  $> 2$ , reflecting an induction by the pathogen, were considered initially. The results show that the highest number of upregulated genes was detected at 24 h post-infection (Table 10). Nevertheless, even at very early infection times (0.5 to 2 hpi), some upregulated genes were also detected. Ninety-five percent of genes upregulated between 0.5 and 2 h showed a very low expression ( $< 10$  FPKM) in the presence of the pathogen. Accordingly, we decided to focus on 24 hpi, when the pathogen has penetrated the plant mesophyll and formed haustorium structures, to identify and select the candidate genes.

Table 10: **Number of soybean genes upregulated during *P. pachyrhizi* infection.** The expression was significantly different between mock and infected tissues (*t. test*:  $p$  value  $< 0.05$  and fold change  $> 2$ ).

Hours post-infection by <i>P. pachyrhizi</i>	Upregulated genes identified
0,5	151
1	112
2	105
24	678

Among the 678 genes upregulated at 24 hpi by *P. pachyrhizi*, we selected the ones induced more than 5 times to eliminate those with a weak induction ratio. We obtained a list of 197 candidate genes, and we retained only those with an absolute expression greater than 10 FPKM

at 24 hpi in the presence of the pathogen. Thus, all genes with low expression (< to 10 FPKM) in the presence of the pathogen were eliminated. With the remaining 37 genes identified, we excluded genes with a high expression level in non-contaminated soybean tissues such as roots, flowers, and pods. To do so, internal data from Bayer CropScience USA (M. Dubald) on gene expression of non-infected soybean tissues (roots, 1<sup>st</sup>, 3<sup>rd</sup> and 5<sup>th</sup> trifoliolate, hypocotyl, growing tips, internode, flowers, and pods) were used. The genes with high expression in non-infected soybean tissues (> 250 FPKM) were excluded (Figure 39-a). This workflow of selection ended with a list of 23 candidate genes (Figure 39-a). The entire set of selected genes were strongly induced at 24 hpi by *P. pachyrhizi* as shown in Figure 39-b

In a second iteration, upregulated genes after a DP7 treatment were identified. Deregulated genes with significantly different expression (*t. test*: p value < 0.05 and) between the DP7 and water treatment in all three replicates were selected. The relative expression  $\left(\frac{\text{expression with DP7 (FPKM)}}{\text{expression with water (FPKM)}}\right)$  of all the deregulated genes was determined, and the relative expression mean of the three replicates was calculated. The deregulated genes were filtered, and only those with a mean relative expression > 2, reflecting an induction by the DP7, were selected. Considering the 23 candidate genes previously identified, we analysed their induction profile following the DP7 treatment. The results are reported in Table 11.

Of the 23 candidate genes, 10 encode a potential function in plant defence according to phytozome (<https://phytozome.jgi.doe.gov/pz/portal.html>): four chitinases, one peroxidase, two genes implicated in the brassinosteroid metabolic pathway, a phosphorylase, an oxidoreductase and a methyltransferase (Table 11).

The aim of our investigation was to find genes exhibiting a low level of expression in mock treatments (*i.e.*, a low basal expression) and high expression in response to rust contamination reflecting a strong induction by the pathogen. All the genes we identified exhibited a low expression level 24 h after a mock treatment, but the majority of them exhibited a moderate expression (between 10 and 40 FPKM) in response to rust contamination (Figure 40 and Table 11).

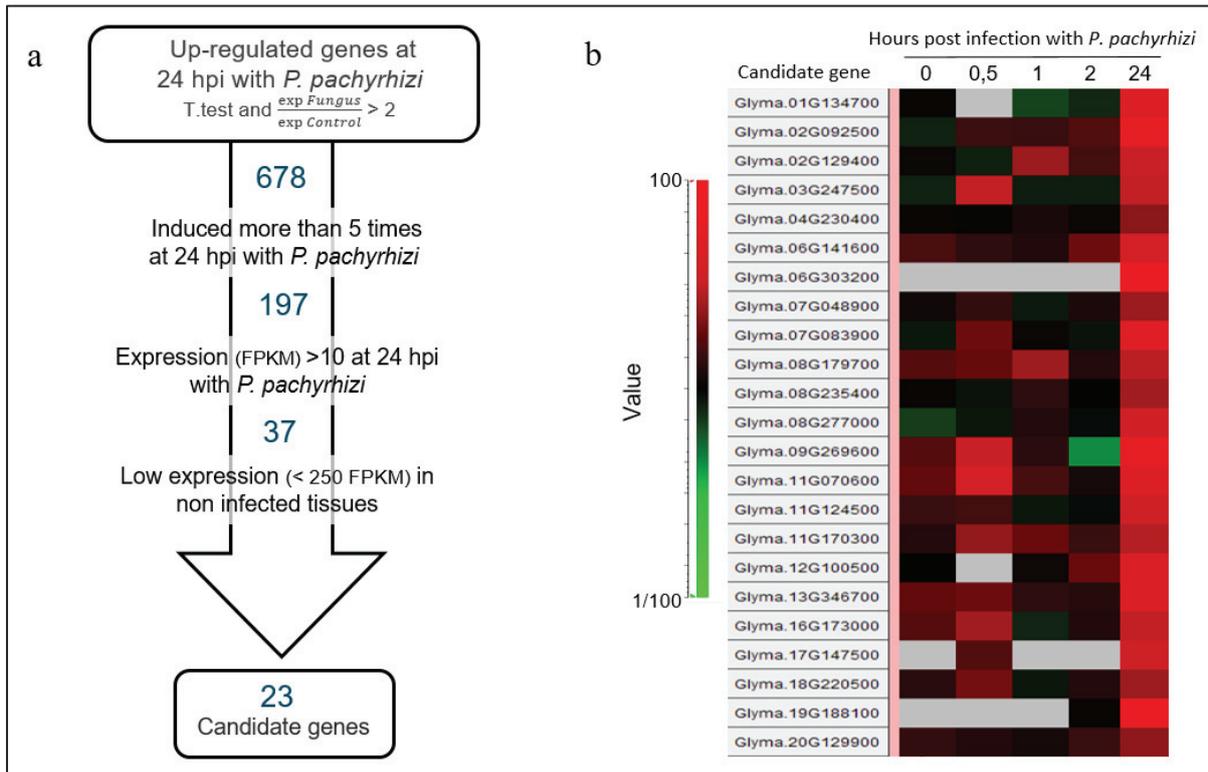


Figure 39: **Candidate gene selection.** a) Workflow for gene selection. Candidate genes highly induced by *P. pachyrhizi* and with low expression in non-infected plant tissues were kept. b) Candidate gene expression during soybean rust infection. Relative expression (compared to the mock). Green: the lowest expression; red: the highest expression; grey: no detection of expression. Internal RNA sequencing data were from Bayer. Analysis was performed with GeneData software.

Three genes (*Glyma.07G083900*, *Glyma.11G170300* and *Glyma.17G147500*) with high expression (> 40 FKM) 24 h after *P. pachyrhizi* inoculation were identified. *Glyma.11G170300* and *Glyma.17G147500* exhibited no induction after treatment with chitin oligosaccharides (the chitinheptaose DP7), unlike *Glyma.07G083900*. We selected *Glyma.11G170300* and *Glyma.17G147500* (in orange in Figure 40 and Table 11) as potentially specifically induced by *P. pachyrhizi* during the early (24 h) stage of infection.

*Glyma.11G124500* (in grey in Figure 40 and Table 11) was also induced by the pathogen and showed no induction after treatment with DP7.

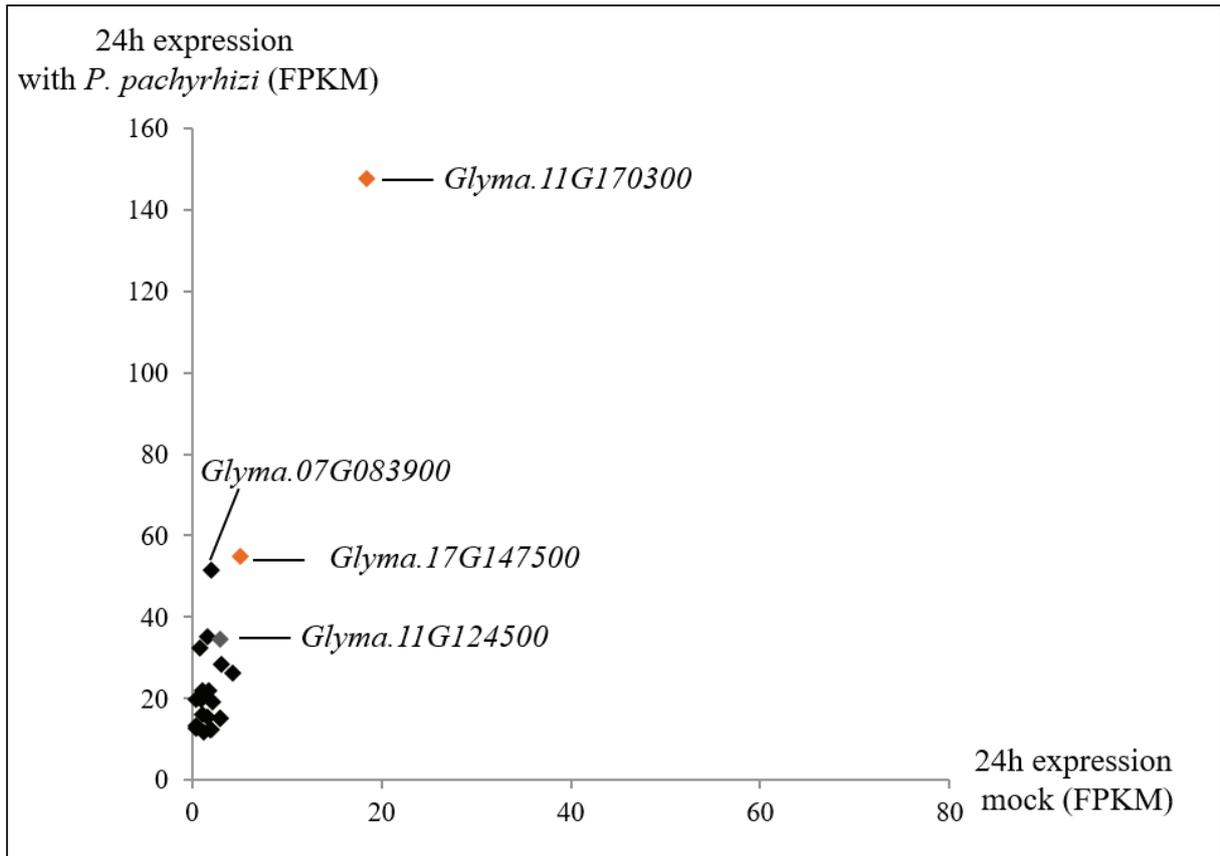


Figure 40: Candidate gene expression after *P. pachyrhizi* and mock inoculation at 24 h. Each point represents a gene. Orange: two genes with the highest expression in response to fungal infection. In grey the *Glyma.11G12450*.

Table 11: **Candidate genes.** Name, annotation and function in plant defence of the 23 genes previously identified. Expression after mock treatment and *P. pachyrhizi* inoculation at 24 h is indicated in FPKM.

name	Expression mock 24 h	Expression with <i>P. pachyrhizi</i> 24h	Induction ratio 24 h	Gene annotation (phytozome)	Function in plant defense	DP7 induction
<i>Glyma.01G134700</i>	1	20	20	Glyceollin biosynthesis II		No
<i>Glyma.02G092500</i>	0.4	13	34	Uncharacterised protein family	...	Yes (0,5h)
<i>Glyma.02G129400</i>	1.2	12	10	...	...	No
<i>Glyma.03G247500</i>	1.7	15	9	Chitinase	PR	No
<i>Glyma.04G230400</i>	3	15	5	...	...	No
<i>Glyma.06G141600</i>	1.1	16	14,5	...	...	Yes (24h)
<i>Glyma.06G303200</i>	0.8	32	40	Isocitrate lyase	...	Yes (24h)
<i>Glyma.07G048900</i>	4	26	6	O-methyltransferase	Gene expression	Yes (24h)
<i>Glyma.07G083900</i>	2	50	25	Phosphorylase	Signalling pathway	Yes (0,5h)
<i>Glyma.08G179700</i>	2.2	19	8.5	Peroxidase	PR	Yes (24h)
<i>Glyma.08G235400</i>	1.9	12	6.5	Trypsin and protease inhibitor		No
<i>Glyma.08G277000</i>	1.8	22	12	Dehydrogenase related		No
<i>Glyma.09G269600</i>	0.4	13	34	Flavonol reductase/cinnamoyl-CoA reductase	Brassinosteroid metabolic pathway	No
<i>Glyma.11G070600</i>	1.6	35	21,5	Dehydratase		No
<i>Glyma.11G124500</i>	3	34	11.3	Chitinase	PR	No
<b><i>Glyma.11G170300</i></b>	<b>18.5</b>	<b>148</b>	<b>8</b>	<b>Asparagine synthase</b>		<b>No</b>
<i>Glyma.12G100500</i>	1.15	21	18	Isocitrate lyase		No
<i>Glyma.13G346700</i>	1.15	22	19	Chitinase	PR	Yes (0,5h)
<i>Glyma.16G173000</i>	3	28	9.3	Chitinase	PR	Yes (0,5h)
<b><i>Glyma.17G147500</i></b>	<b>5.2</b>	<b>55</b>	<b>10.6</b>	<b>Expansin like B1</b>		<b>No</b>
<i>Glyma.18G220500</i>	2	12	6	Flavonol reductase/cinnamoyl-CoA reductase	Brassinosteroid metabolic pathway	Yes (24h)
<i>Glyma.19G188100</i>	0.5	20	40	Oxidoreductase	Oxidative burst	Yes (24h)
<i>Glyma.20G129900</i>	3	15	5	Peptidase		No

...: no annotation. Orange: the genes with the highest expression after *P. pachyrhizi* infection. Grey: *Glyma.11G12450*.

## 2) *GmASP* and *GmEXPLB*: two genes induced during early infection with *P. pachyrhizi*.

*Glyma.11G170300* encodes a protein (*Glyma.11G170300* 1. p) of 579 amino acids with an asparagine synthase domain (PF00733 from amino acid 1 to 560), and it was renamed *GmASP*. Asparagine is implicated in nitrogen metabolism at several developmental phases in plants, including nitrogen mobilization in germinating seeds, nitrogen recycling and flow in vegetative cells in response to biotic and abiotic stresses and nitrogen remobilization from source to sink organs (Gaufichon *et al.*, 2010). Therefore, asparagine synthase plays an important role in these processes by converting aspartate and glutamine to asparagine and glutamate in an ATP-dependent reaction.

The second identified gene, *Glyma.17G147500*, encodes for a protein of 247 amino acids including a lytic transglycolase motif (PF03330 from amino acid 3 to 246) and was reported as expansin-like B1. *Glyma.17G147500* was therefore renamed *GmEXPLB*. Plant expansins and expansin-like molecules are classified into different groups according to their phylogenetic relationship. Expansins participate in the constant assembly, remodelling and disassembly of the cell wall during the plant's lifestyle and contribute to cell wall plasticity. The cell wall acts on cell activity such as differentiation, transport, communication, senescence, abscission, and plant-pathogen interactions and therefore plant growth (Marowa *et al.*, 2016).

### **In planta expression of the selected genes following *P. pachyrhizi* inoculation.**

To validate the chosen genes, the expression of *GmASP* and *GmEXPLB* during the infection of soybean leaves by *P. pachyrhizi* was investigated by RT-qPCR.

In soybean, *GmASP* has 4 homologs (*Glyma.11G171400*, *Glyma.18G061100*, *Glyma.14G195000* and *Glyma.02G228100*) with a very high coding sequences homology (see Annex 1) restricting the choice of primers to a single pair. Due to non-specific PCR amplifications observed with this primer pair, at this stage, no data on *GmASP* expression could be obtained.

*GmEXPBL* expression could be quantified in presence of the pathogen (Figure 41). *GmEXPLB* was induced at 24, 48 and 72 and 240 hpi, with an induction increasing according to the infection time. These results correlate with the *GmEXPLB* expression in transcriptomic data also showing an induction at 24 hpi. Visual symptoms of Asian soybean rust are only visible at 4/5 days after contamination, revealing that the gene was induced before the appearance of the symptoms. *GmEXPLB* was still induced at 240 hpi, when infected leaves were totally chlorotic.

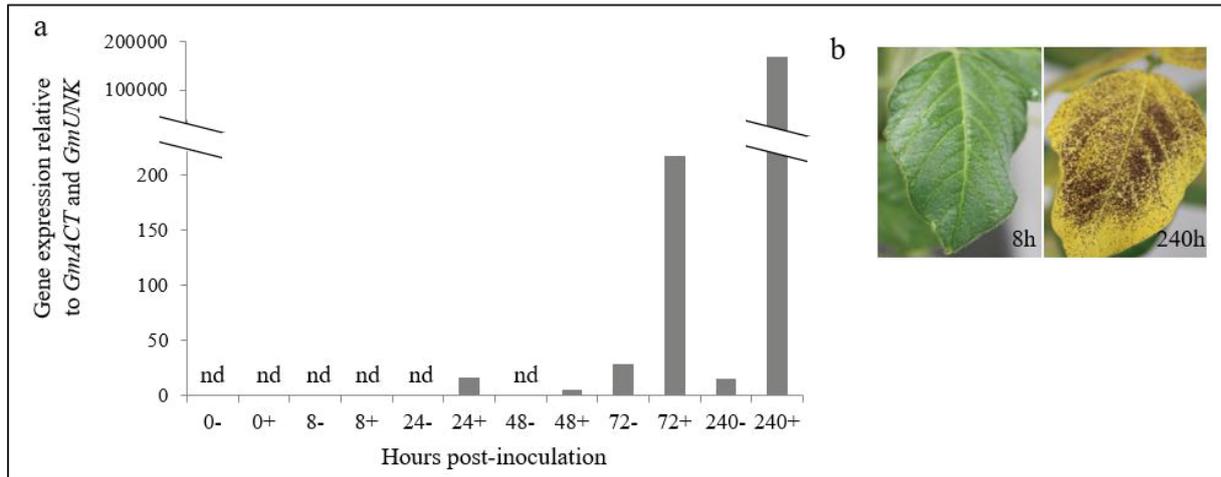


Figure 41: ***GmEXPLB* expression during soybean rust infection.** a) *GmEXPLB* transcript accumulation at 0, 8, 24, 48, 72 and 240 hpi. + *P. pachyrhizi* inoculation and - mock. The actin (*GmACT*, GenBank: NM\_001289231.2) and an unknown protein (*GmUNK*, GenBank: BE330043) encoding genes were used as references (Hirschburger *et al.*, 2015). The same profile was observed in two other biological replicates. nd: not detected. b) Stages of infection of soybean leaves at 8 and 240 hpi.

Both *GmEXPLB* and *GmASP* promoters (1500 bp upstream of the coding sequence of each gene) were selected as candidate promoters induced by the soybean rust. The corresponding sequences (Annexes 2 and 3) of each promoter were synthesized by EUROFINs MWG laboratory.

### 3) The soybean gene *GmCHIT1* is induced at early and late time point of infection by *P. pachyrhizi*.

Published data from Tremblay *et al.*, 2010, 2011, were first used to select *Glyma11G124500* gene induced at a late (10 dpi) time of infection. Among the 23 genes identified using RNA-seq data, *Glyma11G124500* was also identified as induced by *P. pachyrhizi* (in grey in Figure 40 and Table 11) and exhibited no induction by a DP7 treatment. This gene coding for a chitinase was renamed *GmCHIT1*. The identification and characterization of *Glyma11G124500* (*GmCHIT1*) promoter (sequence in Annex 4) are described in a publication submitted to BMC biotechnology and presented from page 149 to 175.

RT-qPCR experiments revealed that this gene was induced at 24 and 240 hpi. Our results correlate with the data obtained by RNAseq and by Tremblay *et al.*, 2010, 2011.

## C. SELECTION OF PROMOTERS FROM THE LITERATURE

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A literature review was performed to select promoters already characterized and identified as potentially inducible by *P. pachyrhizi*. We looked for promoters described as induced by Asian soybean rust. In addition, some promoters are induced by a broad range of pathogens (Himmelbach *et al.*, 2010) and may be considered potential candidates.

### 1) The *gst1* promoter is used in *S. tuberosum* to control *P. infestans*.

In this project, the barnase/barstar system has been selected to develop a new biotechnology approach to control Asian soybean rust. This technology was successfully used by Strittmatter *et al.* (1995) to develop transgenic *S. tuberosum* plants resistant to *Phytophthora infestans*. To drive the *barnase* gene expression, the authors used the *gst1* promoter (pgst1, 273 bp) of *S. tuberosum* activated by *P. infestans* at the infection site at 2 dpi (Figure 42-a).

The *gst1* gene (transcript PGSC0003DMG400002167) encodes for a glutathione S-transferase (GST). Plant GSTs are a family of enzymes that catalyse the conjugation of reduced glutathione to a wide range of substrates, usually resulting in detoxification (Edwards *et al.*, 2000). In plants, GSTs appear to have different functions in primary and secondary metabolism but above all are implicated in cell signalling and biotic/abiotic stress tolerance (Dixon, 2001). In pathogen-infected plants, GSTs play key roles by detoxifying organic hydroperoxides of fatty acids produced from the peroxidation of membranes (Ahn *et al.*, 2016).

In *S. tuberosum*, the *gst1* promoter is activated by different pathogens. It is induced at 3 dpi by the Potato Virus Y (PVY), specifically in tissues in which the virus multiplies (Figure 42-b). This gene is also induced by the potato nematode *Globodera pallida* (Strittmatter *et al.*, 1996), and during a symbiotic interaction, the activity of *gst1* promoter was observed in root tissues colonized by the *Glomus mosseae* mycosis fungus (Strittmatter *et al.*, 1996). *gst1* promoter has been shown not to be induced by abiotic factors such as wounding, heat stress or the absence of light (Martini *et al.*, 1993). However, according to Strittmatter *et al.* (1995), ethylene is an activator of the *gst1* promoter. The basal *gst1* promoter activity in *S. tuberosum* tissues appeared to be restricted to root apices (Figure 42-c) (Strittmatter *et al.*, 1996).

The *gst1* promoter activity following pathogen infection was also studied in apple (Malnoy *et al.*, 2006) and in citrus (Zou *et al.*, 2014a). It has been shown that this promoter is induced by

*Venturia inaequalis* fungus in apple and by *Erwinia amylovora* and *Xanthomonas axonopodis* pv. *citri* bacteria in citrus. Activation of this promoter after wounding was observed in citrus plants and not in apple tissues, suggesting that regulation of the *gst1* promoter depends on the host species.

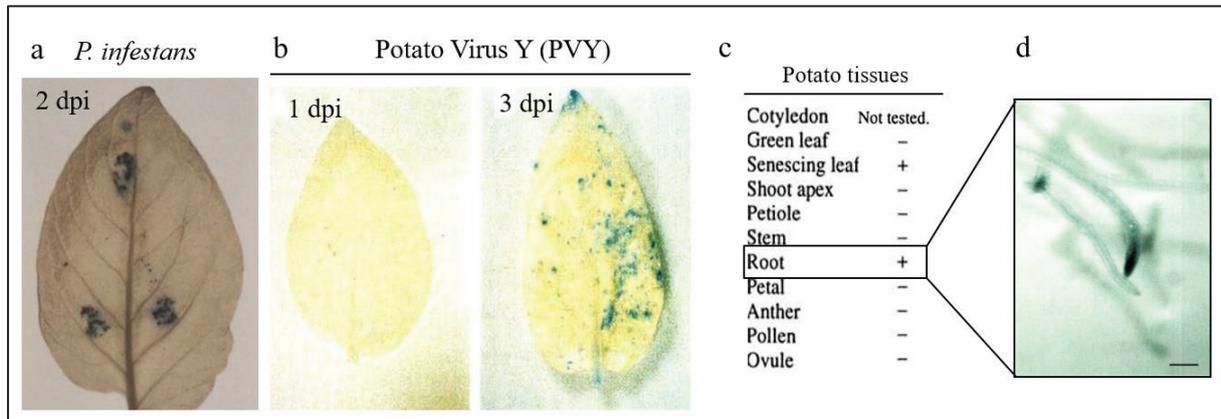


Figure 42: ***gst1* promoter expression in *S. tuberosum* after pathogen infection and in non-infected tissues.** Transgenic *S. tuberosum* harbouring the *pgst1*:GUS fusion. GUS staining of plant leaves 2 dpi with *P. infestans* (a) and 1 and 3 dpi with the potato virus PVY (b). Adapted from Martini *et al.*, 1992, and Strittmatter *et al.*, 1992. c) GUS expression after staining of transgenic plants tissues and (d) an image of non-infected roots. Bar represents 220  $\mu$ m. Adapted from Strittmatter *et al.*, 1992.

We looked for the expression of soybean *gst1* homologues following inoculation of *P. pachyrhizi*. Fourteen homologues of *gst1* were identified in *G. max* according to the OMA Browser (<https://omabrowser.org>), but none of them were strongly induced by Asian soybean rust according to transcriptomic data (see Annex 5) and none of them were present among the 23 candidate genes selected previously.

However, *gst1* promoter is induced by a broad range of pathogens and is functional in different plant species. For these reasons, the *gst1* promoter (273 bp) of *S. tuberosum* was selected as a candidate promoter to be evaluated in soybean. The corresponding sequence (see Annex 6) was synthesized by the EUROFINs MWG laboratory.

## 2) The *GmRIM* promoter is induced by *P. pachyrhizi*

Bibliographic research was conducted to identify promoters induced by Asian soybean rust. The patent WO 2012/127373 A1 (Kuhn *et al.*, 2012) from the BASF Company was the only document describing Asian soybean rust-inducible promoters.

### A soybean peroxidase promoter is described as being induced by *P. pachyrhizi*

In the WO 2012/127373 A1 patent, soybean gene expression was determined by amplified fragment-length polymorphism (AFLP) and microarray. Soybean plants were contaminated

with *P. pachyrhizi*, and leaf epidermis or leaf epidermis and mesophyll cells were harvested at 8, 16 and 112 hpi. The authors identified eight soybean genes upregulated in the presence of the rust in epidermis or both mesophyll and epidermis tissues (Table 12).

Table 12: **Soybean genes upregulated by *P. pachyrhizi* in patent WO 2012/127373.** Name from the last genome version (*Glycine max* 275 William 82) and corresponding putative function (phytozome).

Gene up-regulated by <i>P. pachyrhizi</i>		
Name in WO 2012/127373	Name from last genome version	Putative function
<i>Glyma13g44640</i>	Glyma.13G369100	Kinase
<i>Glyma08g37270</i>	Glyma.08G274400	Serine hydroxymethyltransferase
<i>Gyma04g40860</i>	/	/
<i>Glyma01g33070</i>	Glyma.01G134600	Phenyl transferase
<b><i>Glyma15g05820</i></b>	<b>Glyma.15G052700</b>	<b>Peroxidase</b>
<i>Glyma01g42660</i>	/	/
<i>Glyma17g14320</i>	Glyma.17G134100	Cytochrome P450-like protein related
<i>Glyma01g01510</i>	Glyma.01G011600	Lipase

/ is given when no correspondence was found. Modified from Kuhn *et al.*, 2012.

The induction of these genes during *P. pachyrhizi* infection was confirmed by RT-qPCR, but the results are not presented in the patent. The promoters of the corresponding upregulated genes were selected and used to control the expression of the GUS reporter gene. The majority of the genes were reported as activated in epidermis and in mesophyll tissues (Table 13).

Table 13: **Soybean rust-inducible promoters reported in the patent WO 2012/127373.** Modified from Kuhn *et al.*, 2012.

Feature name	Specificity	Induction by <i>P. pachyrhizi</i>
p-Glyma13g44640 (1047 bp)	Epidermis induced	Yes
p-Glyma08g37270 (2043 bp)	Mesophyll + epidermis induced	Yes
p-Gyma04g40860 (1917 bp)	Mesophyll + epidermis induced	Yes
p-Glyma01g33070 (1921 bp)	Mesophyll + epidermis induced	Yes
<b>p-Glyma15g05820 (1393 bp)</b>	<b>Mesophyll + epidermis induced</b>	<b>Yes</b>
p-Glyma01g42660 (1948 bp)	Mesophyll + epidermis induced	Yes
p-Glyma17g14320 (1607 bp)	Mesophyll + epidermis induced	Yes
p-Glyma01g01510 (2016 bp)	Mesophyll + epidermis induced	Yes

p: promoter; bp: base pair.

One of these inducible promoters (controlling *Glyma.15G052700* expression) is also mentioned in a second BASF patent (US 2014/0137283 A1, Schultheiss *et al.*, 2018). In this document, *Glyma.15G052700* promoter is described as a “rust inducible mesophyll promoter”.

*Glyma.15G052700* was renamed *GmRIM* for *Glycine max Rust Inducible Mesophyll*. *GmRIM* encodes a protein (*Glyma.15G052700* 1. p) of 325 amino acids with a peroxidase motif (PF00141 domain amino acid 7 to 325). Peroxidases are among the pathogen-related (PR) proteins produced in the plant in response to pathogen attacks. These proteins are also implicated in various processes including, for example, root elongation and ROS metabolism (Pandey *et al.*, 2017).

### **Investigation of *GmRIM* expression following *P. pachyrhizi* contamination**

As there is no accurate information on *GmRIM* induction after soybean rust infection (RT-qPCR data are not shown in the WO 2012/127373 A1 patent), we investigated its expression during *P. pachyrhizi* contamination. WT soybean plants were inoculated with the soybean rust, and *GmRIM* expression was followed by RT-qPCR during the infection process at 0, 8, 24, 48, 72 and 240 hpi (Figure 43-a). *GmRIM* was not induced by *P. pachyrhizi* at 24, 48 and 240 hpi. A peak of expression (4.7 times higher in the presence of the rust compared to the non-inoculated plants) was observed at 72 hpi. Uredia were visible only at 4/5 dpi, revealing that the gene was induced before the appearance of the symptoms. At a later time of infection, when the leaf was completely chlorotic (240 hpi), *GmRIM* was no longer overexpressed.

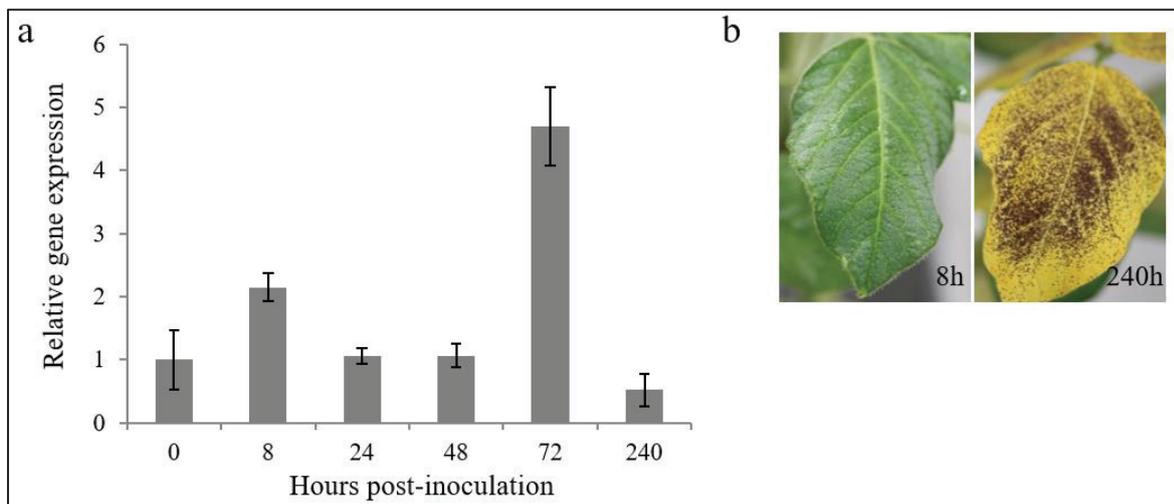


Figure 43: ***GmRIM* expression during soybean rust infection.** a) *GmRIM* transcript accumulation at 0, 8, 24, 48, 72 and 240 hpi compared to that in the mock-treated plants. The actin (GenBank: NM\_001289231.2) and an unknown protein (GenBank: BE330043) encoding genes were used as references (Hirschburger *et al.*, 2015). Three independent biological replicates  $\pm$  standard errors are shown. b) Stages of infection of soybean leaves at 8 and 240 hpi.

We selected *GmRIM* promoter (sequence of 1393 bp upstream of the *GmRIM* start codon reported in Kuhn *et al.*, 2012) as a candidate promoter. The corresponding sequence was synthesized (see Annex 7) by an external laboratory (EUROFINS MWG).



## D. DISCUSSION

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The objective of the study is to evaluate the barnase/barstar system in order to obtain a “proof of concept” for *P. pachyrhizi* control. This approach has been used in *S. tuberosum* and allowed the development of plants resistant to *P. infestans*. In those plants, the barstar was placed under the control of a constitutive promoter and the barnase under the control of the *gst1* promoter induced by *P. infestans*. The same configuration for the evaluation of the system in soybean was kept. Therefore, an Asian soybean rust-inducible promoter is needed to control the barnase expression. The goal was to identify and select promoters to control the barnase expression for the cell death system described above. These promoters must be rapidly activated during *P. pachyrhizi* infection, with the lowest possible background in non-infected tissues and ideally not activated by other stimuli.

Several plant promoters induced by different pathogens have been found (Smirnova and Kochetov, 2015) but few have been reported in soybean. For instance *GmPPO12* (*Glyma04g14361*) promoter has been found to be rapidly and strongly induced by *Phytophthora sojae* and two regions were identified as essential for promoter activity (Chai *et al.*, 2013). In addition Liu *et al.* discovered promoters and associated cis-regulatory elements responsible for the induction by the soybean cyst-nematode *Heterodea glycines* (Liu *et al.*, 2014a).

Rust-inducible promoter have been identified in other plant species. As exemple, the promoter of the *fis1* gene (encoding for an aldehyde dehydrogenase enzyme) from flax (*Linum usitatissimum*) has shown to be locally induced in leaf mesophyll cells surrounding rust (*Melampsora lini*) infection sites (Ayliffe, 2002). The *fis1* promoter also shows endogenous vascular expression. The authors identified the *fis1* homologue in *G. max* (*Glyma.05G029200*) but this gene was not induced by Asian soybean rust according to transcriptomic data. In the literature, except in the patent n° WO 2012/127373 A1, we did not find any publications describing a plant promoter induced by Asian soybean rust.

Five candidate promoters were selected. Three were identified through gene expression analysis and two by bibliographic research (Table 14).

Table 14: **Characteristics of the selected promoters.** Expression of the associated genes under the pathogen infection was observed either in the RNA-seq data by RT-qPCR or in the data from Tremblay *et al.*, 2010 and 2011.

Name	Origin	Associated gene	Length (bp)	Identified from	Induction by <i>P. pachyrhizi</i> in <i>G. max</i>		
					RNAseq (24 hpi)	RT-qPCR	Tremblay et al., 2010 and 2011 (10 dpi)
<b>pGmEXP</b>	<i>Glycine max</i>	<i>GmEXP</i> : Glyma17G147500 Expansin like-B1	1 500	RNAseq data	Yes	Yes (from 24 to 240 hpi)	No
<b>pGmASP</b>	<i>Glycine max</i>	<i>GmASP</i> : Glyma11G170300 Asparagine synthase	1 500	RNAseq data	Yes	On going	No
<b>pGmCHIT1</b>	<i>Glycine max</i>	<i>GmCHIT1</i> : Glyma.11G124500 Chitinase (PR)	3 454	RNAseq data, Tremblay et al., 2010 & 2011	Yes	Yes (from 8 to 240 hpi)	Yes
<b>pgst1</b>	<i>Solanum tuberosum</i>	<i>gst1</i> : Glutathione S-transferase	273	Strittmatter et al., 1995 & 1996, Martini et al., 1993	nd	nd	nd
<b>pGmRIM</b>	<i>Glycine max</i>	<i>GmRIM</i> : Glyma.15G052700 Peroxidase (PR)	1 393	Patent WO 2012/127373 A1	No	Yes (72 hpi)	No

nd: no data

Gene expression analysis using RNA-seq data available in the laboratory was carried out. The number of overexpressed genes with *P. pachyrhizi* at the beginning of the infection was studied. Genes induced by the soybean rust between 0.5 and 2 hpi were identified. During this period, the pathogen only forms a germinated tube on the leaf surface and induced genes reflect the early detection of the pathogen by the plant. At 24 hpi, more genes upregulated by *P. pachyrhizi* were identified. At this time of infection, the appressoria were already formed, the mycelium invaded mesophyll cells and haustorium structures were active. Plant defence signals play a role in the amplification of gene expression (Nürnberg and Kemmerling, 2006). Among the genes induced at 24 hpi, only those with high expression under rust contamination and low expression in non-infected tissues were selected.

Then, two genes (named *GmASP* and *GmEXPLB*) showing the greatest expression in the presence of the pathogen were retained as candidate genes for promoter selection. *GmASP* encodes an asparagine synthase, a protein known to be implicated in nitrogen assimilation and plant defence (Gaufichon *et al.*, 2010). In *Arabidopsis*, the overexpression of an asparagine synthase of pepper (CaASP1) increases defence responses to *Pseudomonas syringae* pv. *tomato* DC3000 and *Hyaloperonospora arabidopsidis* (Hwang *et al.*, 2011). The second selected gene, *GmEXPLB*, encodes an expansin-like B1. Expansins are cell wall remodelling agents that act on cell wall extensibility. Expansin genes (*EXPL2* and *EXPR3* of soybean and *LeEXPA4* and *LeEXPA5* of tomato) have been shown to be upregulated in soybean roots infected with the soybean cyst nematode *Heterodera glycines* and in tomato roots infected with the potato cyst nematode *Globodera rostochiensis* (Marowa *et al.*, 2016).

*GmEXPLB* appeared to be induced by the pathogen from 24 to 240 h. We can hypothesize that a signal perceived during fungal development in the mesophyll, allows the induction of

*GmEXPBL*. A total of 220 genes have been previously identified in Tremblay *et al.*, 2010, and in Tremblay *et al.*, 2011, as upregulated by the pathogen at 240 hpi (see page xx). However, *GmEXPLB* was not present among the 220 genes. The study of the expression of *GmASP* during Asian soybean rust infection must be pursued.

*GmCHIT1* promoter p *GmCHIT1* was selected based on a combination of data from RNA-seq analyses available in the laboratory and the published data from Tremblay *et al.* 2010 and 2011. We showed that this gene is induced at early (8 hpi) at later stages (240 hpi) of infection. The discussion relative to the *GmCHIT1* promoter identification is presented in the submitted publication from page 156 to page 158.

*gst1* promoter was used successfully in the barnase/barstar system in *S. tuberosum* (Strittmatter *et al.*, 1995). *gst1* promoter was activated following nematode (*Globodera pallida*), viral (potato virus Y) and fungal (*P. infestans*, *Glomus mosseae*) infection. A promoter of *A. thaliana* GST (*GSTF8* promoter) is also described as induced by fungal pathogen. *GSTF8* promoter is activated by specific strain of *Rhizoctonia solani* at 2 and 3 dpi in roots regions with very limited mycelium visible (Perl-Treves *et al.*, 2004). The *gst1* promoter has also been shown to be induced by fungal (*Venturia inaequalis*) and bacterial pathogens (*Erwinia amylovora* and *Xanthomonas axonopodis* pv. *Citri*) in apple and citrus (Malnoy *et al.*, 2006; Zou *et al.*, 2014), revealing a non-specific induction by different plant pathogens. This suggests a potential induction of *pgst1* by other organisms such as *P. pachyrhizi*. Therefore, *pgst1* was selected as a heterologous candidate promoter to be evaluated in soybean.

*GmRIM* promoter was described in the WO 2012/127373 A1 patent as being induced in epidermis and mesophyll tissues following soybean rust infection. After penetration in epidermal cells, the Asian soybean rust develops in mesophyll tissues. Consequently, using the *GmRIM* promoter to control the *barnase* expression could allow us to trigger cell death in the infected mesophyll tissues. Other plant peroxidase promoters have been identified as activated by pathogens. For instance, two rice peroxidase promoters (*R2329* and *R2184* promoters) were induced by *Magnaporthe oryzae*. *R2329* promoter was also activated by wounding and the *R2184* promoter by a methyl jasmonate treatment (Sasaki *et al.*, 2007).

*GmRIM* was transiently overexpressed at 72 hpi (RT-qPCR). At this time of contamination, the pathogen has penetrated the plant and colonized the mesophyll cells (Goellner *et al.*, 2010). This finding suggests that *GmRIM* may be transiently activated during pathogen development

in the mesophyll cells. *GmRIM* is not induced by *P. pachyrhizi* at 24 hpi according to the RNA-seq and RT-qPCR data.

Tremblay *et al.*, 2010, and Tremblay *et al.*, 2011, identified upregulated genes at 240 h (10 days) after Asian soybean rust infection. A total of 220 genes upregulated in both experiments were identified, but *GmRIM* was not among them. This finding correlates with the RT-qPCR data showing no induction of this gene at this time of infection (240 hpi).

The activity of these selected candidate promoters will be investigated in order to define the most appropriate to be used in the cell death system for barnase expression control.



## CHAPTER 2

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### EVALUATION OF THE CANDIDATE PROMOTERS



## A. INTRODUCTION

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Five candidate promoters were selected as described in Chapter one (Table 14). Pathogen-inducible promoters are often activated under other conditions than a pathogen infection, such as wounding and/or defence inducers (Muthusamy *et al.*, 2017). As the objective of the barnase/barstar approach is to create a local artificial cell death, the barnase promoter must be strictly controlled: only induced in response to the pathogen and not induced by any other stress. Therefore, this chapter focuses on the evaluation of the candidate promoters to establish their profile of activity in soybean and to select the more appropriate to use in the cell death system.

The GFP reporter gene allowing rapid observations in living tissues over time was selected to follow the promoter activity. The transient system could be considered to rapidly assess the promoter's activity. However, transient transformation of soybean is still difficult to implement and results not reproducible. Therefore, we cannot use it for the evaluation of the promoters. In addition, infection of *N. benthamiana* and *A. thaliana* by the pathogen do not result in a compatible interaction, which prevents us from using those plants for the evaluation of promoter activity after pathogen infection. Therefore, we decided to generate stable transgenic soybeans harbouring the promoter:GFP fusion.

T0 transgenic events were obtained 4 months after transformation, and only those selected for transgene integration were kept for seed production. The activity of the candidate promoters during *P. pachyrhizi* contamination was assessed in T1 soybean events obtained 8 months after the soybean transformation step. Only T1 plants revealing a promoter induced by the pathogen were used to study the promoter activity under other stimuli, *i.e.*, hormonal treatments and wounding. In some case, the promoter activity in non-infected tissues was also observed.

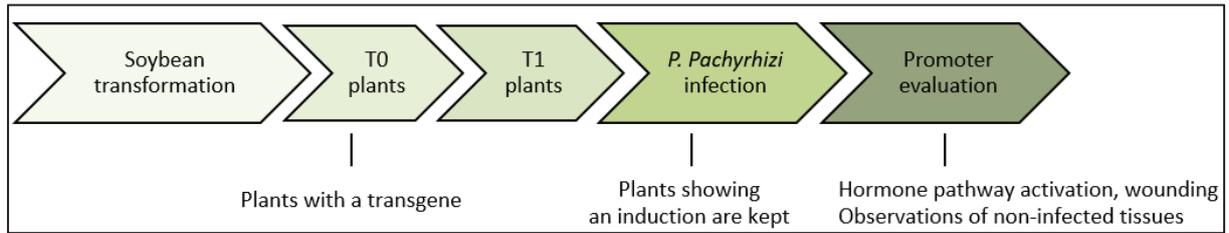


Figure 44: **Workflow for candidate promoter evaluation.** Candidate promoter expression was followed through GFP gene expression. Soybean was transformed with a promoter:GFP cassette. T0 plants with the transgene were kept for seed production. T1 plants were used for evaluation of promoter activity following *P. pachyrhizi* infection. Only plants revealing an induced promoter were used for further investigations.

## B. SOYBEAN TRANSFORMATION FOR PROMOTER EVALUATION

To establish the induction profile of the candidate promoters, fusion promoter:GFP was constructed for each studied promoter (Table 15). The pBay01065 graphical map is presented in Annex 8 as an example. Stable soybean transformations were carried out with the corresponding plasmids.

Table 15: Constructs for promoter evaluation.

Cassette of interest	Construct
p <i>GmRIM</i> :GFP	pBay01065
p <i>gst1</i> :GFP	pBay01108
p <i>GmCHIT1</i> :GFP	pBay00457
p <i>GmASP</i> :GFP	pBay02613
p <i>GmEXPLB</i> :GFP	On going

### 1) Selection of positive transgenic shoots

Regenerated shoots begin to appear one month after the transformation step. T0 events are obtained three months later after one month necessary for development of the shoots (*i.e.*, elongation and rooting) and two months of acclimation in a greenhouse. For all the constructs, soybean regenerated shoots were screened according to the presence of the selectable marker (HPPD-4).

Transgenic shoots were selected through the overexpression of the hydroxyphenylpyruvate dioxygenase (HPPD) protein from *Pseudomonas fluorescens* (HPPD-4). In soybean, HPPD catalyses the formation of homogentisate involved in tyrosine catabolism. HPPD is also the precursor of vitamin E and plastoquinones (Matringe *et al.*, 2005) (Figure 45). Vitamin E is an antioxidant compound, and plastoquinones are implicated in carotenoid biosynthesis and are elements of the chloroplastic electron-transfer chain (Norris *et al.*, 1995). The Tembotrione™ herbicide (TBT) is an HPPD inhibitor, and its application results in a depletion of the plant plastoquinone and vitamin E pools, leading to strong bleaching of the tissues (Matringe *et al.*, 2005).

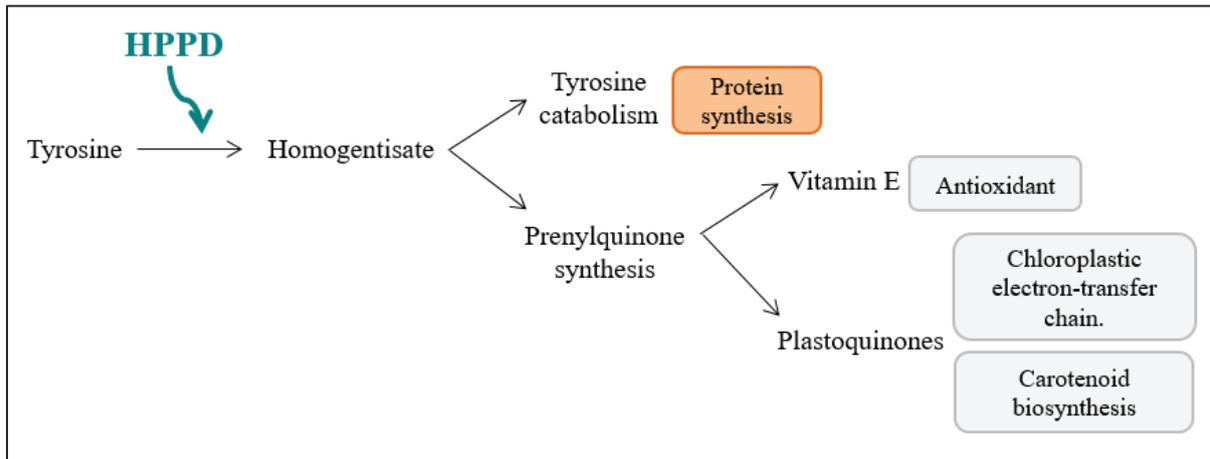


Figure 45: **Involvement of HPPD in prenylquinone biosynthesis and tyrosine catabolism.** From Matringe *et al.*, 2005

In positive transgenic shoots, the overexpression of *HPPD-4* is sufficient to allow the resistance to the TBT. Consequently, on a medium supplemented with the herbicide, the non-transformed shoots are white (TBT sensitive) (Figure 46-a) while the transformed shoots appear green (TBT resistant) (Figure 46-b). Therefore, green transformed shoots were isolated for shoot development. The well-developed shoots were transferred to the greenhouse. The presence of the selectable marker gene in T0 seedlings was confirmed by immunodetection (HPPD-4 lateral flow test, AMAR Immunodiagnostics). Only positive seedlings for this test (Figure 46-c) were selected for further analyses.



Figure 46: **Selection of transformed shoots and seedlings after soybean transformation.** Transformed (a) and non-transformed (b) soybean shoots on TBT medium. c) HPPD-4 immunodetection. One band indicates a negative result (left), and two bands confirm the HPPD-4 overexpression from the transgene (right).

Green shoots growing on TBT medium and positive T0 seedlings for the HPPD-4 lateral flow test were obtained for all the promoter:GFP constructs used. The transformation efficiency was estimated between 1 and 4% (Table 16), revealing a variability of soybean transformation

efficacy. The positive T0 seedlings generated for each construct were transferred to a greenhouse to produce T0 events.

Table 16: **Soybean transformation for promoter evaluation.** The transformation rate corresponds to the T0 events obtained for 100 cotyledons used.

Cassette of interest	Construct	Number of Cotyledons used	Positive shoots on TBT medium	Positive T0 seedlings (HPPD lateral flow test)	T0 events	Transformation rate (%)
<b>pGmRIM:GFP</b>	pBay01065	530	69	18	18	3,7
<b>pgstI:GFP</b>	pBay01108	513	100	20	20	3,8
<b>pGmCHIT1:GFP</b>	pBay00457	397	109	11	11	2,7
<b>pGmASP:GFP</b>	pBay02613	610	47	5	5	0,8

## 2) Evaluation of the GFP signal

The basal GFP fluorescence on leaves of 5 to 7 T0 events by construct was observed and compared to that of a WT plant (Figure 47).

In the pGmRIM:GFP, pgstI:gfp, and pGmASP:GFP plants, two different profiles were observed. Some events showed a fluorescence intensity similar to that of the WT. It is possible that the GFP gene might not be properly expressed in these events (gene not transferred, truncated sequence or a position effect), and therefore, they were not selected for further studies. Other events exhibited a visible GFP signal with a fluorescence intensity higher than that of a WT plant, reflecting a functional GFP. These events were selected for T1 seed production.

For the T0 events carrying the pGmCHIT1:GFP construct, all the tested plants showed a fluorescence intensity similar to that of the WT. Assuming that the basal activity of the GmCHIT1 promoter in T0 leaves is too low to be detected with a dissection scope, all the T0 events obtained were kept for further observations on T1 plants.

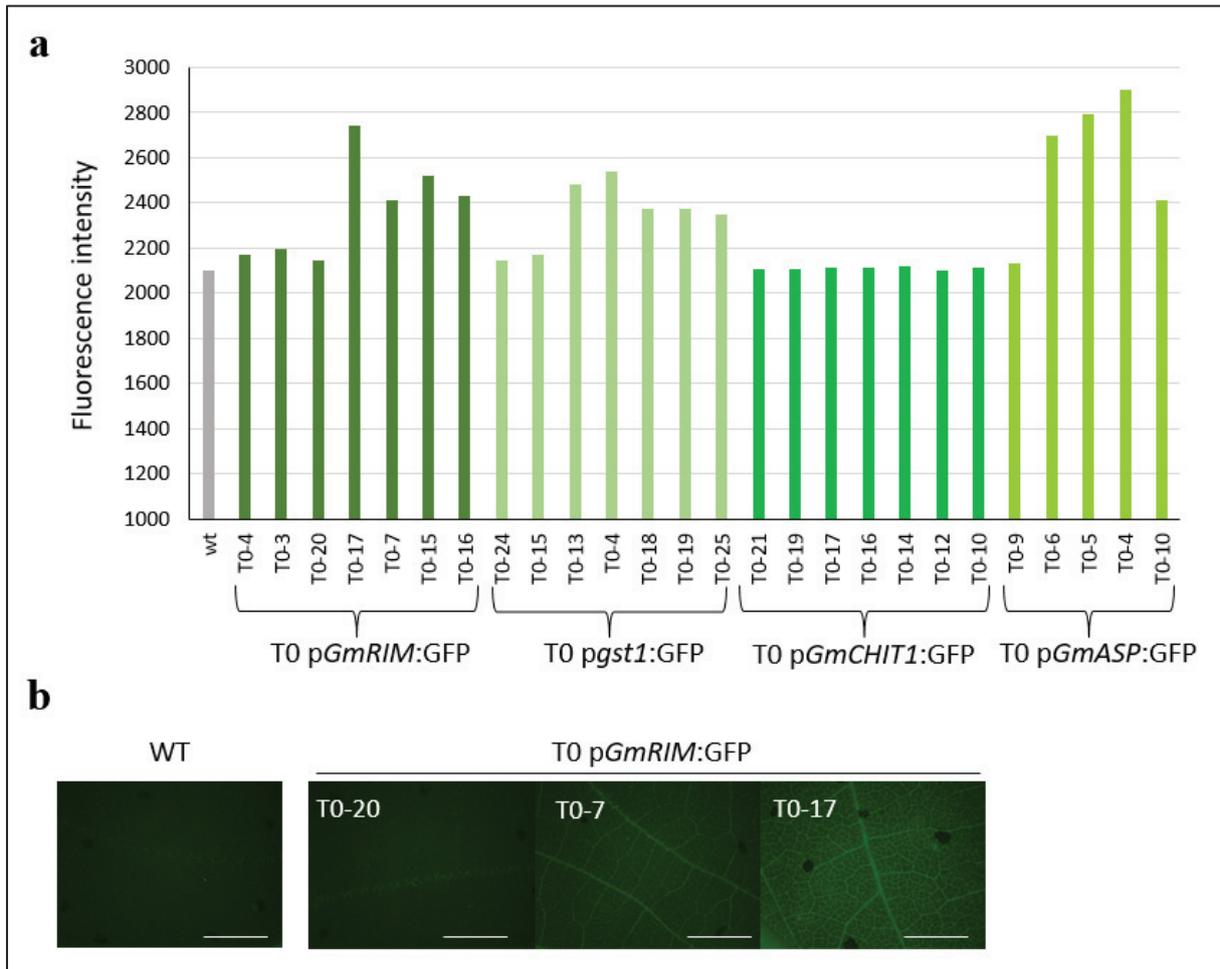


Figure 47: **GFP fluorescence in T0 events.** a) Fluorescence intensity in leaves of a WT plant and T0 events with the *pGmRIM:GFP*: *pgst1:GFP*, *pGMCHIT1:GFP* and *pGmASP:GFP* cassettes. b) Example of images obtained with *pGmRIM:GFP* T0 events and a WT plant. Observation was performed with a dissection scope (Leica Z16 APO) under GFP filter. Scale bar: 5 mm.

### C. PROMOTER ACTIVITY UNDER SOYBEAN RUST INFECTION

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Induction in response to *P. pachyrhizi* infection is the most important criterion to select a promoter used to control the barnase expression. Therefore, the promoter activity in response to soybean rust infection was analysed by detection of fluorescence in the different transgenic soybean plants described above.

#### 1) *pGmRIM* and *pGmASP* activity in response to *P. pachyrhizi* contamination

*pGmRIM:GFP* plants from 3 independent T1 events (137, 138 and 139) were inoculated with soybean rust. No clear augmentation of the GFP signal was observed at 72 hpi around the penetrated cells in the 3 events tested. A basal GFP signal was nevertheless observed in the mock-treated leaves (Figure 48-a), revealing a basal expression of the *GmRIM* promoter in soybean leaves.

T0 events with the *GmASP* promoter controlling the GFP were obtained later at the end of the project. Consequently, T0 *pGmASP:GFP* plants were exceptionally directly used for investigation of *GmASP* promoter activity in response to *P. pachyrhizi* infection instead of T1. Detached leaves of 3 T0 plants were inoculated by the pathogen, but no clear augmentation of the GFP signal was observed around the penetrated cells in the 3 T0 events tested (Figure 48-b).

The *GmRIM* and *GmASP* promoters did not show a clear induction by the Asian soybean rust at 72 hpi. Therefore, they were not selected for further studies.

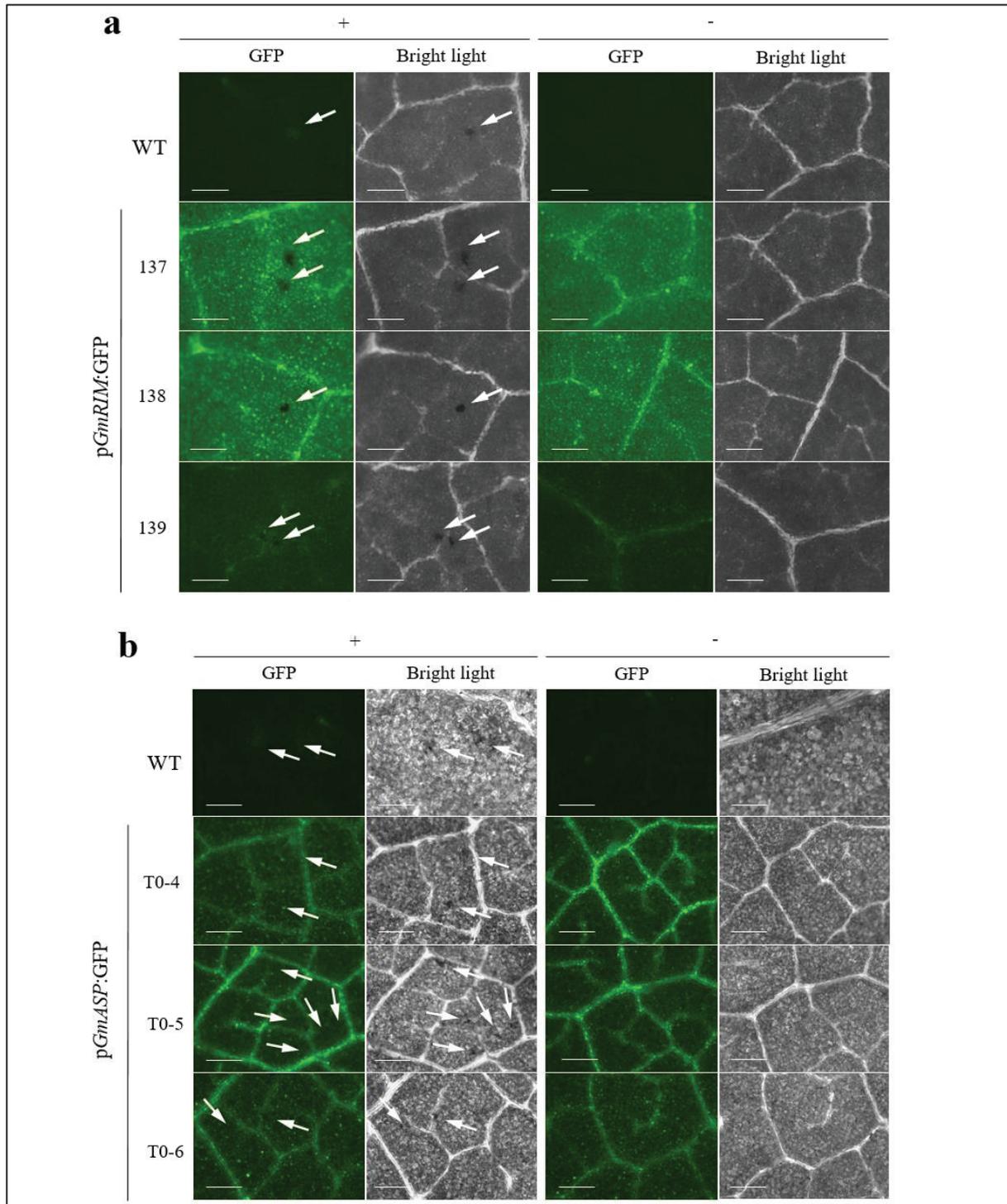


Figure 48: *GmRIM* and *GmASP* promoter activity in soybean leaves 72 h after *P. pachyrhizi* infection. a) Leaves of three T1 *pGmRIM:GFP* events (137, 138, 139) were observed using a dissection scope (Leica Z16 APO) under GFP filter and bright light. *P. pachyrhizi* inoculation (+) and mock (-). The same results were obtained for three plants by event. b) Leaves of 3 T0 *pGmASP:GFP* events (T0-4, T0-5, T0-6). Observation was performed using a dissection scope (Leica Z16 APO) under GFP filter and bright light. *P. pachyrhizi* inoculation (+) and mock (-). Arrows indicate the inoculation spots. Scale bars represent 200  $\mu$ m.

## 2) *pGmCHIT1* and *pgst1*: two promoters induced in response to *P. pachyrhizi* contamination.

*P. pachyrhizi* spores were sprayed on three T1 *pGmCHIT1*:GFP plants. Fluorescence surrounding the infection spots was observed in three independent T1 events (129, 131, 133) (Figure 50). Under these observation conditions (magnification higher than for the observations of T0 leaves), a basal expression in non-infected leaves was observed mainly in the veins of the three T1 events.

*pgst1*:GFP T1 seeds from three T0 events were sown, but for one event, the seeds did not germinate properly and no T1 plants were obtained. Therefore, only two independent T1 events were tested. An increase of the GFP signal globally restricted to the inoculation spots was well observed on one T1 event (148) and a bit less on the other T1 event (147) at 72 hpi. A strong basal GFP signal was observed on the tissues following the mock treatment (Figure 50).

GFP fluorescence was confirmed by a Western blot analysis of the three *pGmCHIT1*:GFP T1 events. An accumulation of GFP protein was observed at 72 hpi under *P. pachyrhizi* infection (Figure 49), correlating with the increase of fluorescence observed around the infection spots.

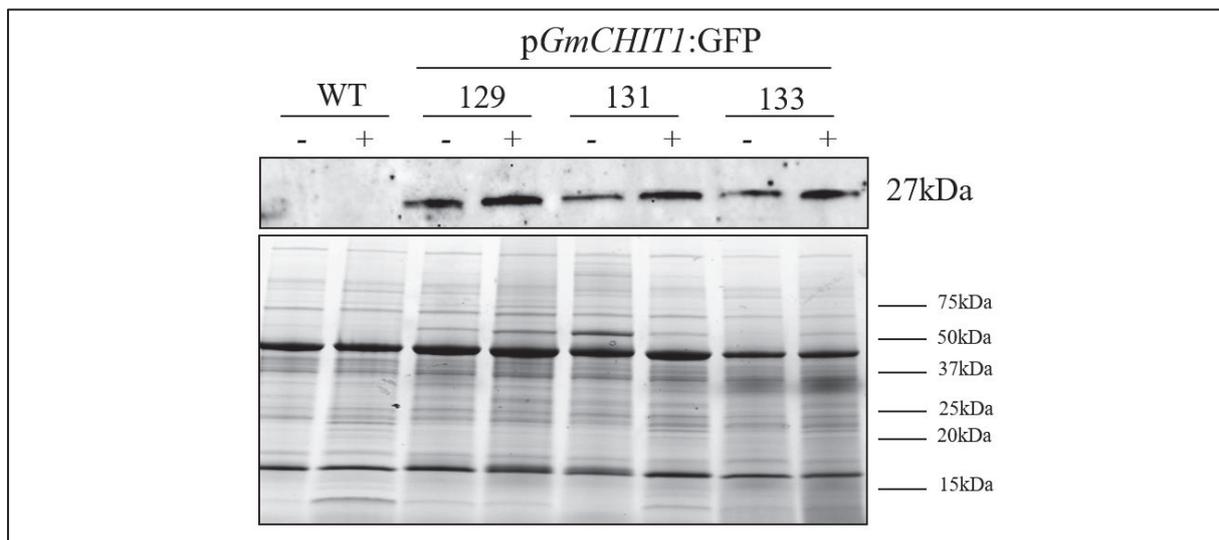


Figure 49: **Detection of GFP in *pGmCHIT1*:GFP soybeans.** Above, detection of GFP protein by immunoblotting with an antibody raised against the GFP. Below, homogenous loading was checked on the gel by Strain Free detection technology (Bio-Rad, US).

The *GmCHIT1* and *gst1* promoters were activated by the soybean rust 3 days post-contamination. Both possess a basal expression in non-infected leaves. The *gst1* promoter is expressed in all leaves and the *GmCHIT1* promoter mainly in leaf veins. These two promoters were selected for further study.

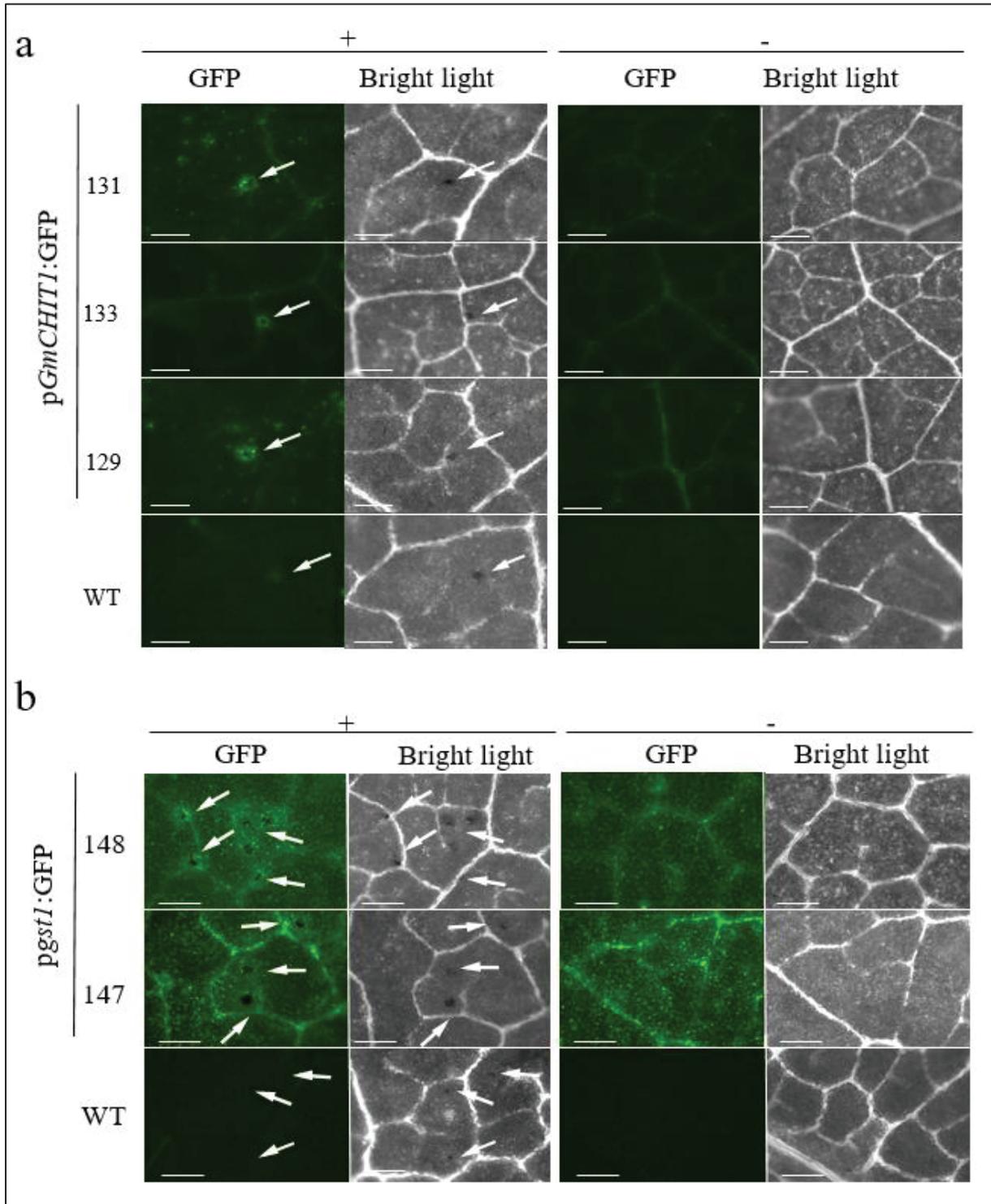


Figure 50: *GmCHIT1* and *gst1* promoter activity in soybean leaves 72 h after *P. pachyrhizi* infection. a) Leaves of three T1 *pGmCHIT1*:GFP events (129, 131, 133) and two T1 *pgst1*:GFP events (148, 147) were observed using a dissection scope (Leica Z16 APO) under GFP filter and bright light. *P. pachyrhizi* inoculation (+) and mock (-). Arrows indicate the inoculation spots. Scale bars represent 200  $\mu$ m. The same results were obtained for three plants by events.

## D. *GmCHIT1* AND *gst1* PROMOTER ACTIVITY

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Further investigations of *GmCHIT1* and *gst1* promoters activity were conducted on the event 131 of p*GmCHIT1*:GFP and 148 of p*gst1*:GFP.

### 1) Activity of the *GmCHIT1* and *gst1* promoter at early infection time

A new *P. pachyrhizi* inoculation was realized on p*GmCHIT1*:GFP and p*gst1*:GFP T1 plants and the GFP fluorescence was observed at 24 and 48 hpi in order to evaluate the induction of the promoter during the early steps of infection before 72 hpi (Figure 51). No induction of the *gst1* promoter was detected at 24 and 48 h post *P. pachyrhizi* contamination. On the other hand, an increase of fluorescence intensity was observed surrounding the infection spots at 24 and 48 hpi on p*GmCHIT1*:GFP plants, revealing that *GmCHIT1* promoter was induced at early time of infection (Figure 51). A confocal microscopy study was conducted on p*GmCHIT1*:GFP plants at 24 hpi after fungal germ tubes and appressoria staining. Confocal microscopy images revealed that the GFP fluorescence was detectable around the fungal appressoria (Figure 5 page 153).

### 2) Activity of the *GmCHIT1* and *gst1* promoter in response to hormone treatments

To precise if the *GmCHIT1* and the *gst1* promoters could be induced by stimuli other than fungal contamination, different hormonal treatments were performed.

In the laboratory, soybean plants expressing the GFP under control of the PDF1.2 promoter (plants named *PDF1.2*) have been generated. *A. thaliana* PDF1.2 promoter has been shown to be induced by jasmonate and ethylene (Manners *et al.*, 1998). Experimental conditions for ethylene and jasmonate induction were validated with these T2 homozygous plants used as positive controls. As expected GFP fluorescence strongly increased from 24 to 72 hours after coronatine (methyl jasmonate analogue) and ACC (ethylene precursor) treatments in *PDF1.2* detached leaves (Figure 52). Contrary to the *PDF1.2* plants, fluorescence intensity did not change after coronatine or ACC spray on p*GmCHIT1*:GFP plant (event 131) showing that p*GmCHIT1* was not induced by these hormonal treatments. A low increase of the GFP signal after a spray of ACC was observed at 72 hours in p*gst1*:GFP plants (event 148) (Figure 52).

However, the fluorescence intensity did not change after coronatine treatment on those plants revealing that *gst1* promoter was induced by ACC but not by coronatine treatment.

Fluorescence intensity was unchanged after SA exposure in p*GmCHIT1*:GFP and p*gst1*:GFP plants (Figure 53-a). PDF1.2 promoter is not induced by SA. Therefore, to test the effect of this treatment, the expression of three *PR* genes (*GmPR1*, *GmPR2* and *GmPR3*) was followed by RT-qPCR in the leaves of p*GmCHIT1*:GFP plants (event 131). Only a low induction of *GmPR3* (2-fold change compared to mock) was detected in the leaves treated with SA (Figure 53-b). However, unlike in Mazarei *et al.*, (2007) *GmPR1* was not induced after our salicylic acid treatment. It is unclear at this stage whether the results reflect a lack of efficacy of salicylic acid treatment or an insensitivity of p*GmCHIT1* and p*gst1* to this hormone.

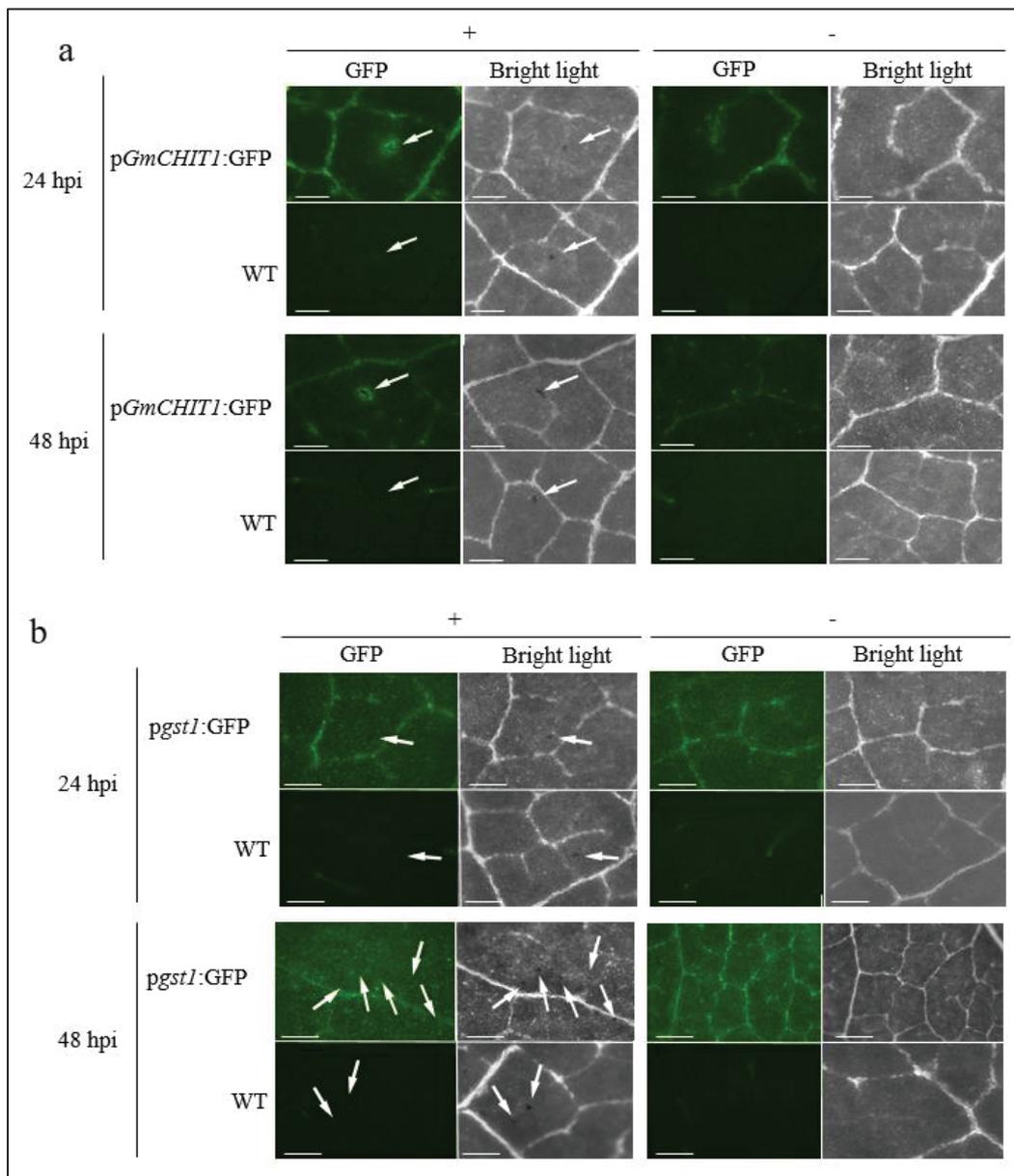


Figure 51: *GmCHIT1* and *gst1* promoter activity, 24 and 48 h after *P. pachyrhizi* infection. Leaves of a) p*GmCHIT1*:GFP (event 131) and b) p*gst1*:GFP (event 148) plants were observed using a dissection

scope (Leica Z16 APO) under GFP filter and bright light at 24 and 48 hpi. *P. pachyrhizi* inoculation (+) and mock (-). Arrows indicate the inoculation spots. Scale bars represent 200  $\mu$ m. The same results were obtained for three plants by events.

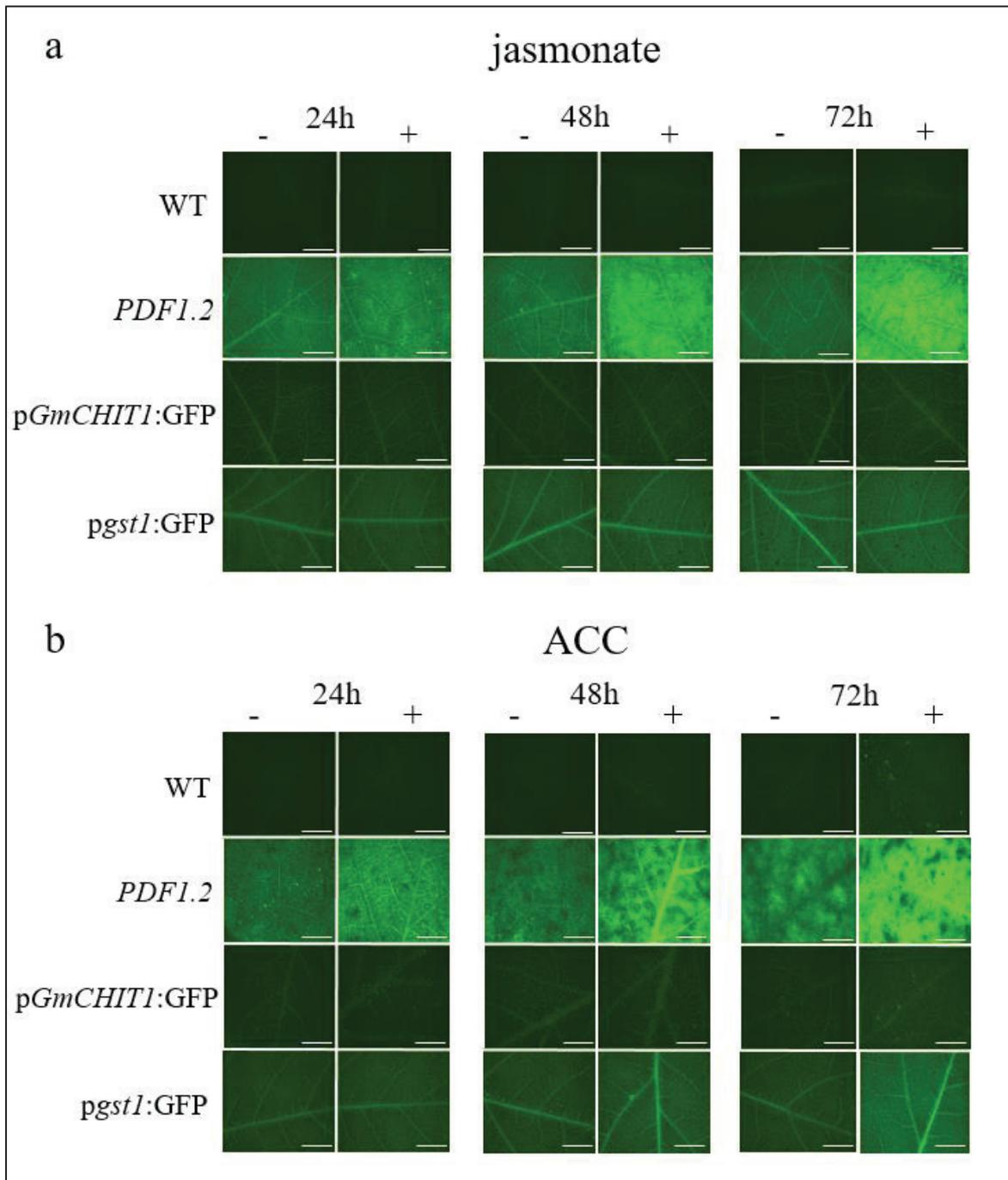


Figure 52: ***GmCHIT1* and *gst1* promoter expression following hormonal signalling activation.** GFP fluorescence p*GmCHIT1*:GFP (line 131), p*gst1*:GFP (line 148) *PDF1.2* (p*PDF1.2*:GFP) or WT detached leaves following hormones (+) or mock (-) treatments. Treatment with coronatine (a) and ACC (b). Scale bars represent 5mm. Observations were realized at 24, 48 and 72 hours after hormonal treatment with a dissection scope (Leica Z16 APO) under GFP filter. Same results were obtained for three plants per events.

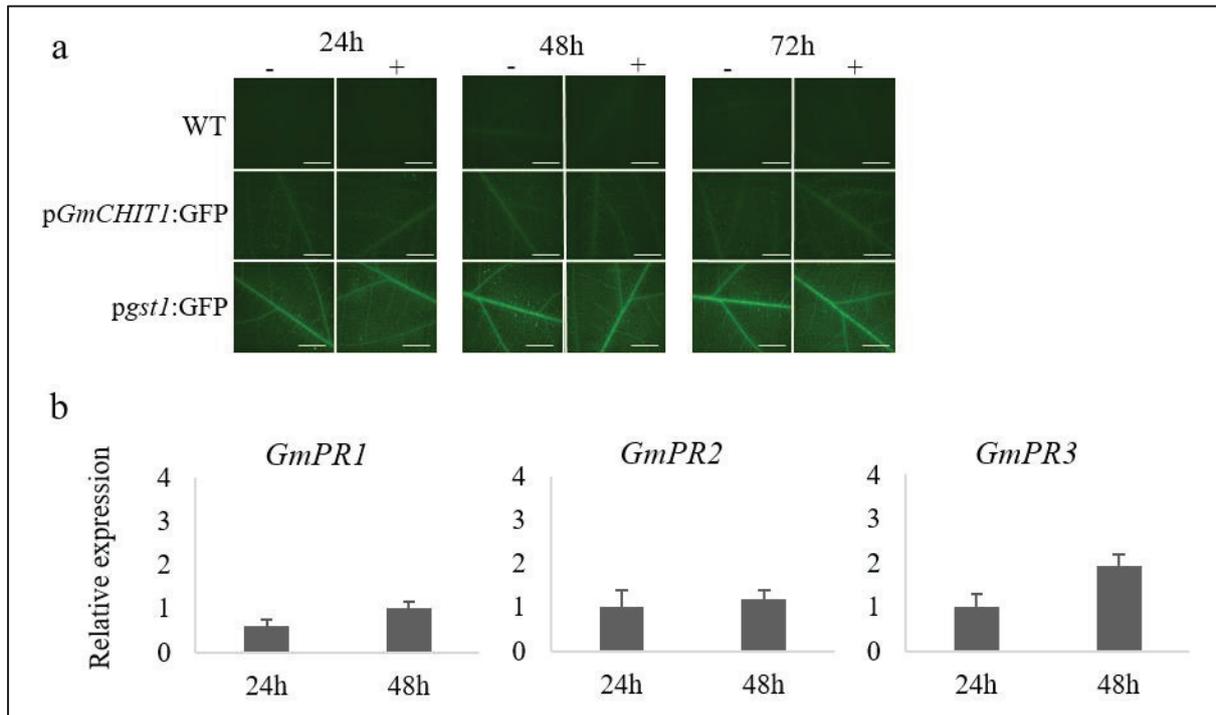


Figure 53: ***GmCHIT1* and *gst1* promoter expression following SA signalling activation.** a) GFP fluorescence p*GmCHIT1*:GFP (line 131), p*gst1*:GFP (line 148) or WT detached leaves following hormones (+) or mock (-) treatments. Scale bars represent 5mm. Observations were realized at 24, 48 and 72 hours after hormonal treatment with a dissection scope (Leica Z16 APO) under GFP filter. Same results were obtained for three plants by events. b) *GmPR1* (GenBank: BU5773813), *GmPR2* (GenBank: M37753), *GmPR3* (GenBank: AF202731) expression in p*GmCHIT1*:GFP (event 131) after SA treatment. Transcript accumulation at 24 and 48 hours compared to that in the mock-treated plants. The actin (GenBank: NM\_001289231.2) and an elongation factor (GenBank: NM\_001249608.2) encoding genes were used as references. Three independent biological replicates  $\pm$  standard deviations.

### 3) Activity of the *GmCHIT1* and *gst1* promoter in response to wounding

The *GmCHIT1* and *gst1* promoters response was monitored after a mechanical wounding. In p*GmCHIT1*:GFP plants (event 131), a small GFP fluorescence was observed at 24 hours post wounding limited to the wounded area and still visible at 72 hours after the injury (Figure 54). *GmCHIT1* promoter appeared to be induced by wounding with no propagation to adjacent tissues. In p*gst1*:GFP plants (event 148), no increase of GFP fluorescence was observed, revealing that this promoter is not induced by a wounding.

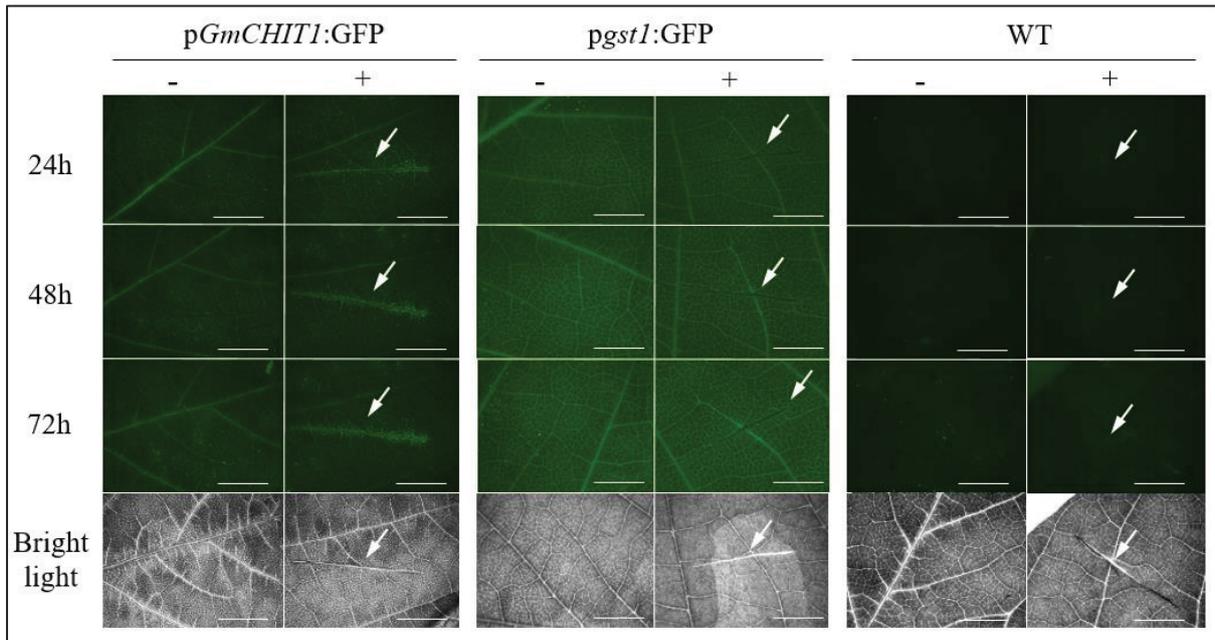


Figure 54: ***GmCHIT1* and *gst1* promoter response after wounding.** GFP fluorescence in wounded (+) or control (-) detached leaves from p*GmCHIT1*:GFP (event 131) and p*gst1*:GFP (event 148) transgenic soybeans and WT plants. Bar-scale represents 5mm. Observations at 24, 48 and 72 hours after wounding with a dissection-scope (Leica Z16 APO) under GFP filter and bright light. Arrows show the wounded part. Same results were obtained for three plants by events.

The results obtained for the *GmCHIT1* promoter and its expression in soybean tissues are presented in the submitted publication “Identification and characterization of a new soybean promoter induced by *Phakopsora pachyrhizi*, the causal agent of Asian soybean rust”. The article also presents the identification of the *GmCHIT1* promoter.

## Identification and characterization of a new soybean promoter induced by *Phakopsora pachyrhizi*, the causal agent of Asian soybean rust.

Cabre L<sup>1</sup>, Peyrard S<sup>2</sup>, Sirven C<sup>2</sup>, Gilles L<sup>2\*</sup>, Pelissier B<sup>2</sup>, Ducerf S<sup>2#</sup>, Poussereau N<sup>1</sup>

<sup>1</sup> Functional Genomics of Plant Pathogenic Fungi, UMR 5240 Microbiology Adaptation Pathogeny, CNRS-UCB-INSA-Bayer SAS, 14 Impasse Pierre Baizet BP 99163, 69263 Lyon Cedex 09

<sup>2</sup> Bayer SAS, Crop Science Division, 14 Impasse Pierre Baizet, BP 99163, 69263 Lyon Cedex 09, France.

\*Present address: Laboratoire de Reproduction et Développement des Plantes, ENS de Lyon 46, allée d'Italie, 69364 LYON Cedex 07, France.

#Corresponding author: Sophie Ducerf: [sophie.ducerf@bayer.com](mailto:sophie.ducerf@bayer.com)

### ABSTRACT

**Background:** *Phakopsora pachyrhizi* is a biotrophic fungal pathogen responsible for the Asian soybean rust disease causing important yield losses in tropical and subtropical soybean-producing countries. *P. pachyrhizi* triggers important transcriptional changes in soybean plants during infection, with hundreds of genes being either up- or downregulated.

**Results:** Based on published transcriptomic data, we identified a chitinase gene, referred to as *GmCHIT1*, upregulated in the first hours of infection. We first confirmed this early induction in our studies and then investigated the promoter of *GmCHIT1*. Transgenic soybean plants expressing the green fluorescence protein (GFP) under the control of the *GmCHIT1* promoter were generated. Following contamination with *P. pachyrhizi*, GFP fluorescence was detected in a limited area around appressoria, the fungal penetration structure. Fluorescence of leaves from p*GmCHIT1*:GFP transgenic plants was also observed after mechanical wounding. No variation in fluorescence of p*GmCHIT1*:GFP transgenic plants was detected after a treatment with an ethylene precursor and a methyl jasmonate analogue.

**Conclusion:** We identified a soybean chitinase promoter induced by *P. pachyrhizi* at 24 h post-inoculation in the first infected soybean leaf cells. This promoter is also induced by wounding but not by hormonal treatments. Our results on the strong induction of *GmCHIT1* promoter by *P. pachyrhizi* infection contribute to the development of a strategy for disease control using biotechnological approaches.

**KEYWORDS:** Soybean, *Phakopsora pachyrhizi*, induction, chitinase, promoter, GFP

## BACKGROUND

Rusts are among the most damaging crop diseases, causing very severe losses in crop yield <sup>1,2</sup>. In particular, Asian soybean rust is the most destructive foliar disease of soybean (*Glycine max* (L.) Merr.) <sup>3</sup> and is caused by the biotrophic fungus *Phakopsora pachyrhizi* Syd. & P. Syd. Initially localized in Asia, *P. pachyrhizi* has spread across the world and reached the South American continent in the 2000s, where it is causing important yield losses. Brazil, one of the leading soybean-producing countries, is impacted by the disease each year. Highest damages on grain harvest between 2007 and 2014 reached 571.8 thousand tons, e.g., 6% of the national production <sup>4</sup>. *P. pachyrhizi* directly penetrates the epidermal cells of its hosts. Once in the mesophyll, haustoria are formed and proliferation of hyphae occurs. Approximately 5-8 days post infection, uredinia appear on the abaxial side of the leaves and new urediniospores are released, leading to contamination of healthy plants through airborne spore dissemination <sup>5</sup>. Symptoms are characterized by tan-coloured lesions and chlorosis of the leaves. In the most severe cases, defoliation and quick maturation of soybean with a reduction of seed size and weight can be observed in a few days after initial infection <sup>6,7</sup>.

Today, the control of *P. pachyrhizi* is essentially based on fungicidal treatments. Demethylation inhibitors (DMIs) impairing sterol biosynthesis, as well as succinate dehydrogenase inhibitors (SDHIs) and quinone outside inhibitors (QoIs), blocking mitochondrial respiration, are the most commonly used fungicides <sup>8,9</sup>. However, the repetitive use of molecules with these three modes of action and the fungicide adaptation capability of the pathogen have resulted in a decrease in treatment efficacy <sup>4</sup>. However, genetic resistance of soybean to *P. pachyrhizi* is well documented and could be seen as an alternative to the use of pesticides. Thus far, seven dominant R genes, named *Rpp1* to *Rpp7*, have been identified <sup>10-14</sup>. However, these resistance genes are only effective against specific isolates of *P. pachyrhizi* <sup>15</sup> and the resistance conferred by these genes can be easily overcome, making breeding solutions very challenging <sup>16</sup>. Today, no soybean cultivars resistant to most of the rust isolates are available. In this context, biotechnological approaches are foreseen as alternative solutions to control Asian soybean rust <sup>9,17</sup>.

A common strategy in plant engineering for disease resistance is to overexpress a defence-related gene placed under the control of a constitutive promoter. However, permanent and high ectopic expression of such genes can impact the plant's fitness and development <sup>18</sup>. These challenges can be overcome by using a pathogen-inducible promoter allowing transgene expression only when and where it is needed. Pathogen-inducible promoters are composed of cis-regulatory elements that contain binding sites for transcription factors and/or regulatory proteins. These elements that regulate gene expression patterns can be activated by different stimuli <sup>19</sup>. As a consequence, pathogen-inducible promoters are often induced by other stimuli such as wounding and/or hormones. Many pathogen-inducible promoters have been studied in different plants <sup>20-22</sup>, but very few have been reported in soybean. For instance, *GmPPO12* (*Glyma04g14361*) promoter has been found to be rapidly and strongly induced by *Phytophthora sojae*, and two regions were identified as essential for promoter activity <sup>23</sup>. In addition, Liu *et al.* discovered several cis-regulatory elements responsible for the induction by the soybean cyst-nematode *Heterodera glycines* <sup>24</sup>.

Plant reactions to pathogen attacks involve the activation of a set of genes coding for different proteins. Among them, pathogenesis-related (PR) proteins are produced and highly

accumulated<sup>25</sup>. Chitinases represent a subset of pathogenesis-related proteins. These enzymes have the ability to randomly hydrolyse beta-1,4-glycoside bonds of chitin, a major component of the fungal cell wall. The resulting chitin fragments act as a potent pathogen-associated molecular pattern (PAMP) that induces PAMP-triggered immunity<sup>26</sup>. Plant chitinases have also been shown to be implicated in the defence against insects; in response to abiotic stresses such as cold, drought or metal toxicity; and in development<sup>27,28</sup>. Plant chitinases belong to families 18 and 19 of the glycosyl hydrolases and are further classified into 5 classes based on primary structure (class I to class V)<sup>26</sup>.

In this publication, we report the identification and characterization of the soybean chitinase promoter p*GmCHIT1* that we selected from a set of transcriptomic data. This promoter drives both early and late overexpression of its gene upon *P. pachyrhizi* infection. Its specificity to fungal exposure over general hormonal pathway activation and abiotic stress was evaluated through the generation of stable transgenic soybeans harbouring a p*GmCHIT1*:GFP fusion. To our knowledge, this is the first characterization of a soybean promoter inducible by Asian soybean rust.

## RESULTS

### **The soybean chitinase gene *GmCHIT1* is induced by Asian soybean rust**

Several transcriptomic data on soybean gene expression during *P. pachyrhizi* infection have been generated and published. In 2010, Tremblay *et al.* used DNA array to analyse gene expression in the palisade and mesophyll cells infected by the pathogen. They identified 685 upregulated genes 10 days after soybean rust inoculation (dpi), and most of them were related to plant defence response and metabolism<sup>29</sup>. In 2011, they used next-generation sequencing (NGS) to analyse soybean gene expression patterns in leaves and described 1713 genes upregulated 10 dpi, with many of them encoding proteins involved in metabolism and transport<sup>30</sup>. Since upregulated genes are a potential source of inducible promoters, we searched for genes upregulated in both experiments. We identified 220 common upregulated genes, and a ranking of these genes according to their fold changes was determined for each experiment (see additional file 1: Table S1). Among the commonly upregulated genes, one-quarter (26%) were associated with metabolism function (Figure 1). Two other functions were also well represented, with 18% of genes implicated in signal transduction and 12% annotated as transporters. Eleven plant defence-related genes representing 5% of the commonly upregulated genes were also identified. Among them, two genes annotated as predicted chitinase (*Glyma.13G346700* and *Glyma.11G124500*) were shown to be highly induced at 10 dpi and were also upregulated 24 h post-infection, in agreement with SoyKB data (<http://soykb.org/>). According to internal transcriptomic data (unpublished data), *Glyma.11G124500* showed no induction after treatment with a chitin oligosaccharide (the chitinheptaose DP7), in contrast to *Glyma.13G346700* (see additional file 2: Figure S1). We selected *Glyma.11G124500* as potentially specifically induced by *P. pachyrhizi* during early (24 h) and late stages of infection (10 days).

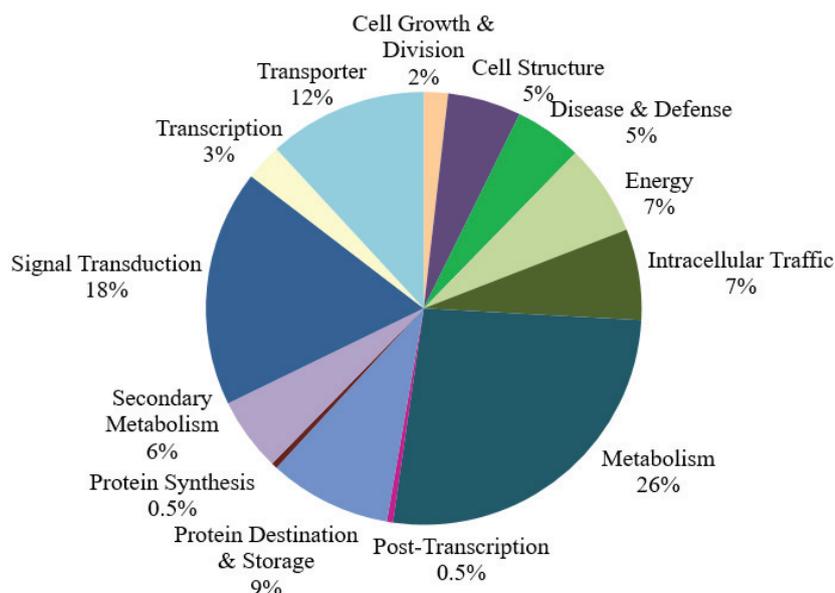


Figure 1: **Percentage of physiological functions of the 220 genes upregulated 10 days post-contamination with *P. pachyrhizi*.** Soybean genes identified in Tremblay *et al.*, 2010, and Tremblay *et al.*, 2011.

*Glyma.11G124500* is located on chromosome 11 and includes a coding sequence of 705 bp containing two exons: a 5'UTR of 57 bp and a 3'UTR of 217 bp. This gene encodes a protein (*Glyma.11G124500* 1. p) of 235 amino acids with a glycosyl hydrolase motif of family 19 (PF00182 domain from amino acid 38 to 235) and was reported as a chitinase. This putative function was confirmed by a phylogenetic tree (Figure 2). *Glyma.11G124500* was therefore renamed *GmCHIT1*.

### ***In planta* expression of *GmCHIT1* following *P. pachyrhizi* contamination**

Expression of *GmCHIT1* during infection of soybean leaves by *P. pachyrhizi* was monitored by RT-qPCR. *GmCHIT1* was expressed as early as 8 h after *P. pachyrhizi* inoculation (2.5-fold compared to the mock treatment, Figure 3a), and its expression increased during infection reaching 6-7-fold compared to the mock treatment at 1-3 dpi (days post-infection). *GmCHIT1* highest expression level (300-fold compared to healthy leaves) was observed at a late stage of infection when the inoculated leaves were totally chlorotic and covered with sporulating uredinia (10 dpi) (Figure 3a, b). In our conditions, no visual symptoms were observed at 8 hpi (hours post-inoculation) and uredia appeared at 6/7 dpi, revealing that the gene was induced before symptom apparition (Figure 3-b). Consequently, we selected the *GmCHIT1* promoter as a candidate induced by *P. pachyrhizi*.

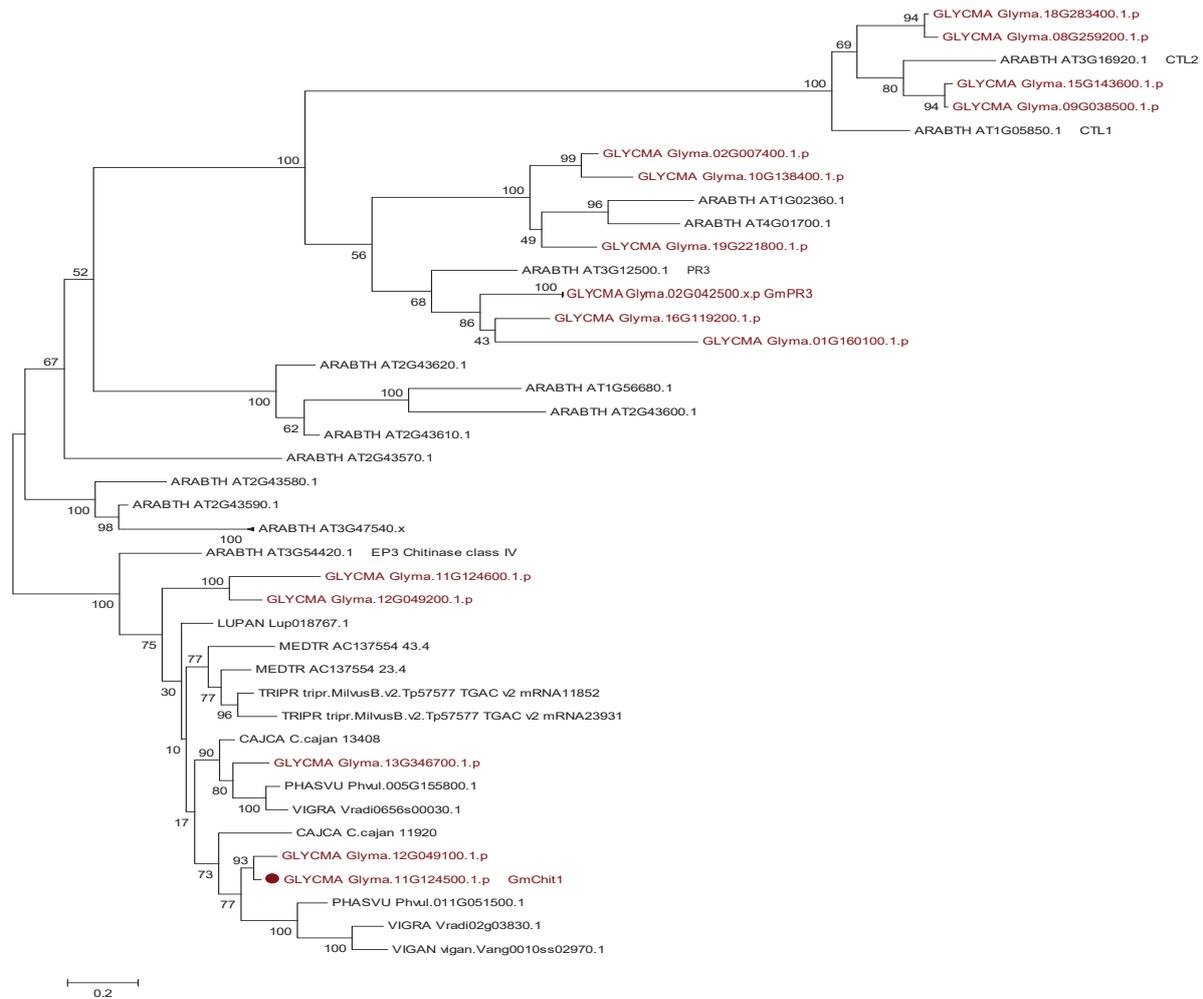


Figure 2: **Phylogenetic analysis of the GmCHIT1 protein (Glyma.11G124500 1. p) based on plant homologous, *A. thaliana* and *G. max* proteins containing the glycosyl hydrolase motif of family 19 (PF00182 domain).** The phylogenetic tree was generated by PhyML (Guindon and Gascuel, 2003) after alignment with Clustal Omega (Sievers et al., 2011) (maximum likelihood method). The numbers at the nodes represent the bootstrap values. Red: *G. max* proteins; the red bullet points correspond to GmCHIT1. GLYCMA: *Glycine max*; ARABTH: *Arabidopsis thaliana*; LUPAN: *Lupinus angustifolius*; MEDTR: *Medicago truncatula*; CAJCA: *Cajanus cajan*; TRIPR: *Trifolium pratense*; PHASVU: *Phaseolus vulgaris*; VIGRA: *Vigna radiata*; VIGAN: *Vigna angularis*.

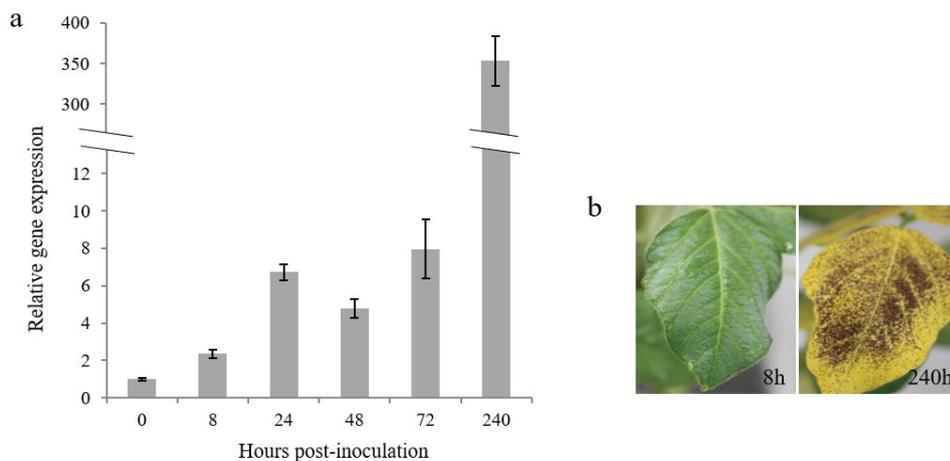


Figure 3: ***GmCHIT1* expression during *P. pachyrhizi* infection.** a) *GmCHIT1* transcript accumulation at 0, 8, 24, 48, 72 and 240 hpi compared to that in the mock-treated plants. The actin (GenBank: NM\_001289231.2) and an unknown protein (GenBank: BE330043) (Hirschburger *et al.*, 2015) encoding genes were used as references. Three independent biological replicates  $\pm$  standard errors are shown. b) Stages of infection of soybean leaves at 8 and 240 hpi.

### **Analysis of the activity of the *GmCHIT1* promoter in response to *P. pachyrhizi***

To study the expression of the *GmCHIT1* promoter, a fragment of 3454 bp upstream of the coding sequence containing regulatory regions was selected as a pathogen-induced candidate promoter. Indeed, analysis of this sequence with PLACE software <sup>31</sup> revealed several cis-regulatory elements related to pathogen infection (see additional file 3: Table S2 and additional file 4: Figure S2). Twenty-one W boxes (TGAC) <sup>32</sup> and 10 GT1 boxes (GAAAAA) <sup>33</sup> were identified. Five MYB recognition elements (GGATA)<sup>34</sup> were also found as well as two auxin (TGTCTC and KGTCCCAT) <sup>35</sup> and two gibberellic acid-responsive elements (CAACT) <sup>36</sup>.

The activity of the *GmCHIT1* promoter following *P. pachyrhizi* contamination was evaluated via the generation of stable transgenic soybeans. The promoter region was fused to the GFP reporter gene (p*GmCHIT1*:GFP), and transgenic plants were selected. *P. pachyrhizi* spores were sprayed on the plants, and fluorescence surrounding the infection spots was observed at 24 and 72 hpi in three independent p*GmCHIT1*:GFP lines (129, 131, 133) (Figure 4a). GFP signal was also observed in leaf veins in the absence of the fungal infection (control), revealing a basal expression of the promoter in fully developed 3-week-old soybean plants. GFP-fluorescence was confirmed by a Western blot analysis, revealing the accumulation of GFP-protein at 72 hpi (Figure 4b). A confocal microscopy study was conducted on line 131 at 24 hpi after fungal germ tubes and appressoria staining. Confocal microscopy images revealed that the GFP fluorescence was particularly detectable around the fungal pathogen and more precisely around appressoria, the fungal penetration structures (Figure 5).

### **Activity of the *GmCHIT1* promoter in different soybean tissues**

To determine the tissue specificity of the chitinase promoter, GFP fluorescence of plants from the 131 line was investigated in roots, young leaves and flowers of non-infected plants. p*CsVMV*:GFP plants containing the strong constitutive Cassava Vein Mosaic Virus promoter were used as a positive control. As expected, a strong GFP fluorescence was observed in all analysed tissues of plants transformed with p*CsVMV*:GFP, whereas no GFP signal was detected in WT soybean plants (Figure 6). In the case of plants transformed with p*GmCHIT1*:GFP (line 131), a light GFP signal was detected in primary and some lateral roots. While GFP expression was observed in veins of developed leaves (Figures 4, 7 and 8), no signal was detectable in young leaves at this magnification (Figure 6). This low detection of GFP could be considered the baseline expression of the *GmCHIT1* promoter.

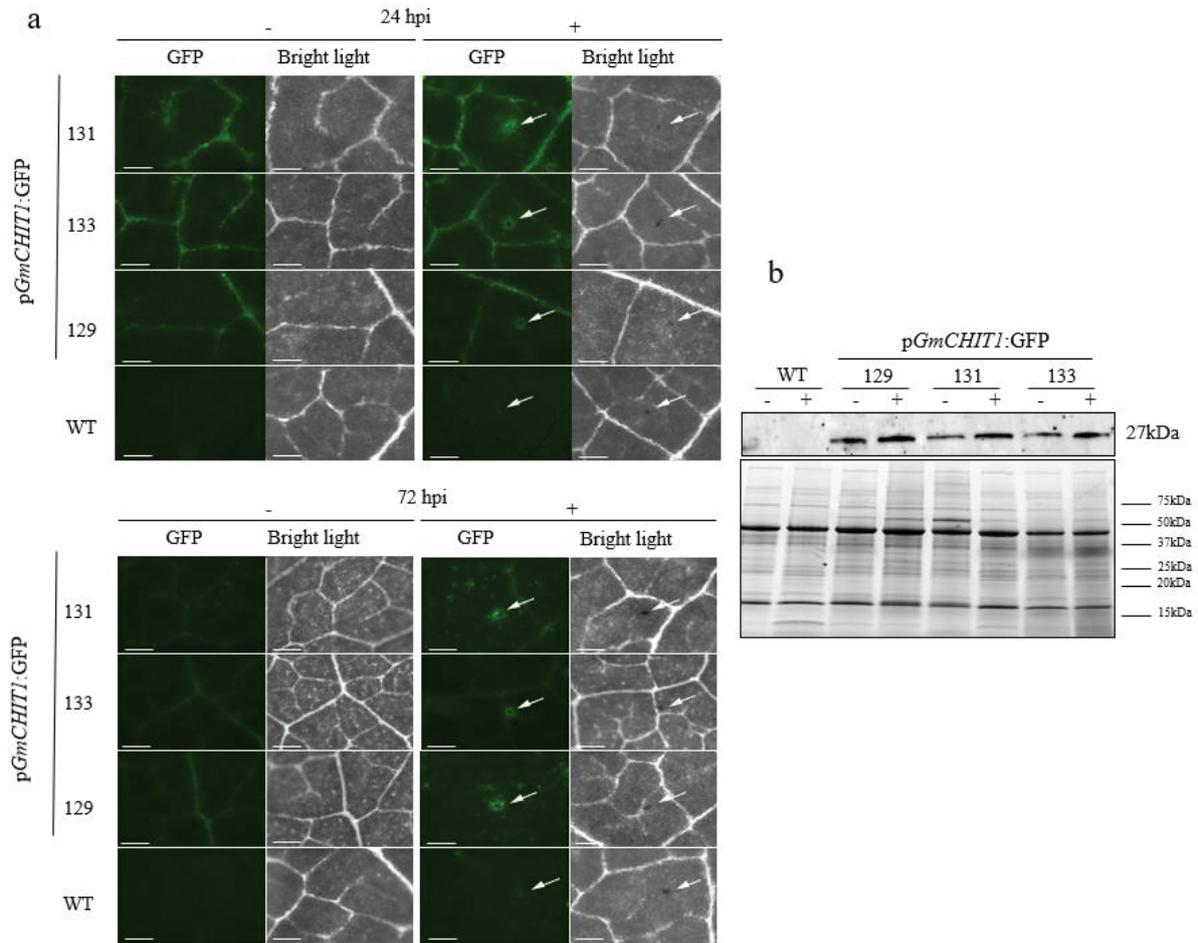


Figure 4: **Detection of GFP fluorescence in stable transgenic soybeans.** (a) Leaves of three T1 lines (129, 131 and 133) with the *pGmCHIT1*:GFP construction were observed using a dissection scope (Leica Z16 APO) under GFP filter and bright light at 24 and 72 h after fungal contamination. *P. pachyrhizi* inoculation (+) and mock (-). Arrows indicate the inoculation spots. Scale bars represent 200  $\mu$ m. (b) Above, detection of GFP protein by immunoblotting with an antibody raised again the GFP. Below, homogenous loading was checked on the gel by Strain Free detection technology (Bio-Rad, US).

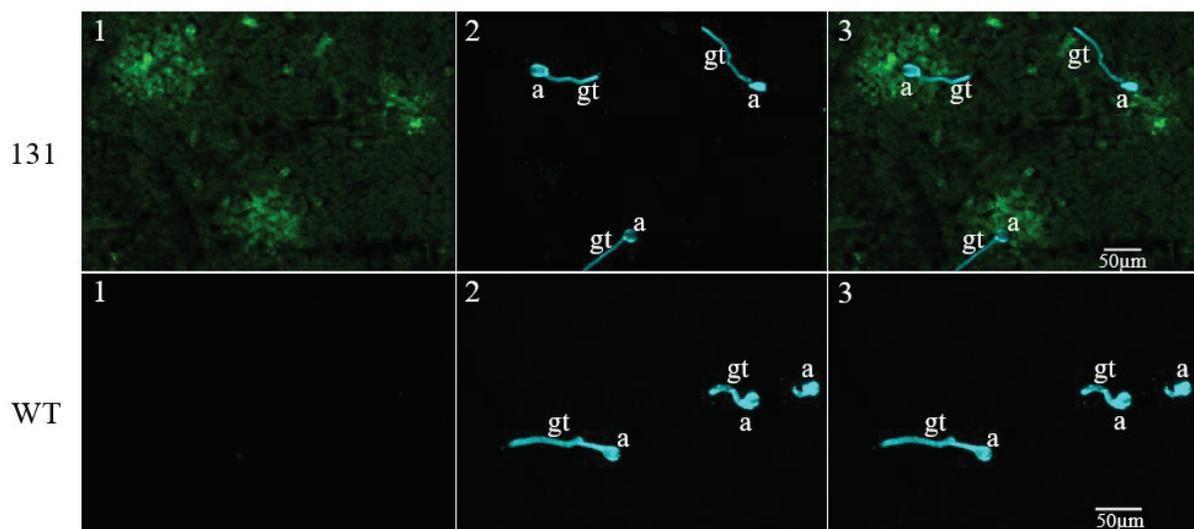


Figure 5: **GFP induction around *P. pachyrhizi* appressoria at 24 hpi.** Confocal images (z-stack projection) were observed 24 hpi. Fungal structures on the leaf surface are stained in blue with calcofluor. a: appressorium, gt: germ tube. Picture 1: GFP detection. Picture 2: calcofluor staining. Picture 3: merging of pictures 1 and 2. The observations were conducted on 131 (*pGmCHIT1*:GFP) and WT plants.

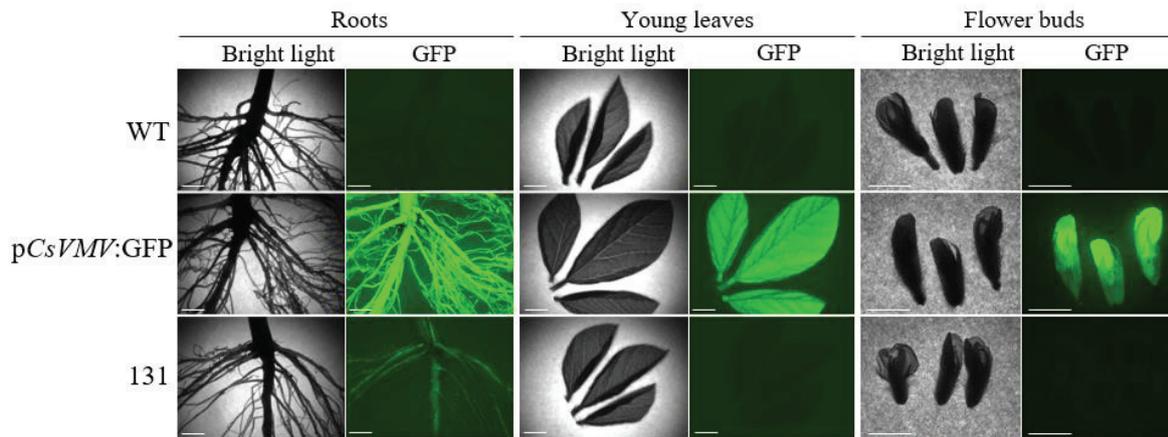


Figure 6: **GFP activity mediated by *GmCHIT1* promoter (line 131) in soybean tissues (roots, leaves, flower buds).** Plants transformed with pCsVMV:GFP were used as a positive control. Scale bars represent 5 mm. Pictures were taken with a dissection scope (Leica Z16 APO) under GFP filter and bright light.

### Activity of the *GmCHIT1* promoter in response to hormone and wounding treatments

To evaluate the activity profile of the *GmCHIT1* promoter, different hormonal treatments were performed on plants of line 131. *A. thaliana* *PDF1.2* promoter has been shown to be induced by jasmonate and ethylene<sup>20</sup>. Consequently, p*PDF1.2*:GFP soybean plants (named *PDF1.2*) were used as positive controls for these investigations. As expected, GFP fluorescence strongly increased from 24 to 72 h after coronatine (methyl jasmonate analogue) and 1-aminocyclopropane-1-carboxylic acid (ACC, ethylene precursor) treatments (Figure 7a-b). Fluorescence intensity did not change after coronatine or ACC spray on p*GmCHIT1*:GFP plants (Figure 7a-b), showing that p*GmCHIT1* is not induced by these hormonal treatments. Fluorescence intensity remained unchanged after salicylic acid (SA) exposure in p*GmCHIT1*:GFP plants (Figure 7c). To test the effect of this treatment on p*GmCHIT1*, the expression of three *PR* genes (*GmPR1*, *GmPR2* and *GmPR3*) was followed by RT-qPCR in the leaves of 131 plants (additional file 5: Figure S3)<sup>37</sup>, but only a low induction of *GmPR3* (2-fold change compared to mock) was detected in the leaves treated with SA. Lastly, *GmCHIT1* promoter response was monitored after mechanical wounding. A small GFP fluorescence was observed at 24 h post-wounding limited to the wounded area and still visible at 72 h after the injury (Figure 8). The *GmCHIT1* promoter appeared to be induced by wounding with no propagation to adjacent tissues.

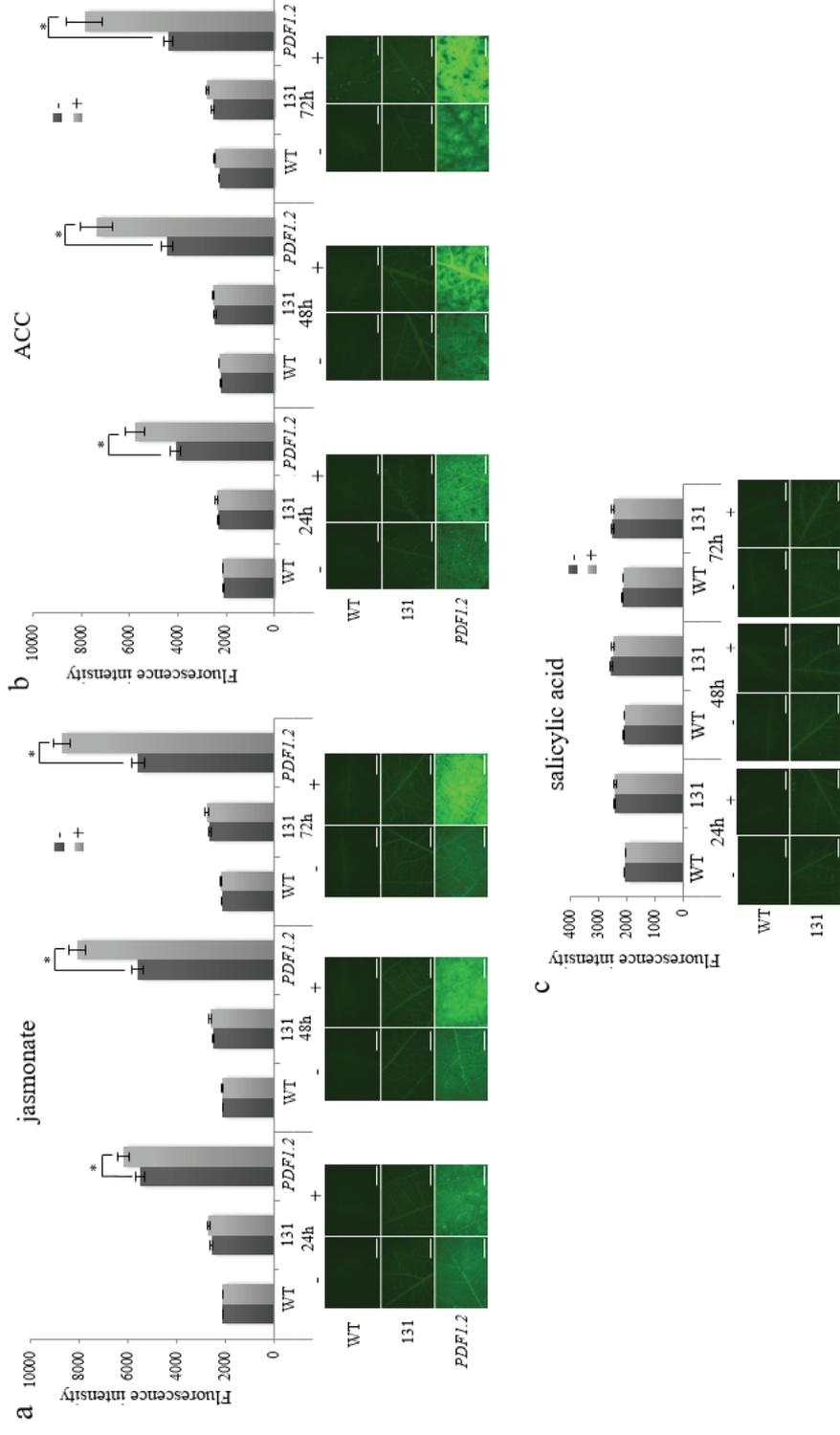


Figure 7: *GmCHIT1* promoter expression following hormonal signalling activation. GFP fluorescence in 131 line (p*GmCHIT1*:GFP), *PDF1.2* (p*PDF1.2*:GFP) or WT detached leaves following hormones (+) or mock (-) treatments. Treatment with coronatine (a), ACC (b) and salicylic acid (c). Graphics represent the fluorescence intensity. Mean of 20 biological replicates  $\pm$  standard errors. \*: significant difference between treated (+) and untreated (-) leaves determined by a t-test ( $p < 0.05$ ). Representative images of the observed fluorescence are shown under the graphs. Scale bars represent 5 mm. Observations were performed at 24, 48 and 72 h after hormonal treatment with a dissection scope (Leica Z16 APO) under a GFP filter.

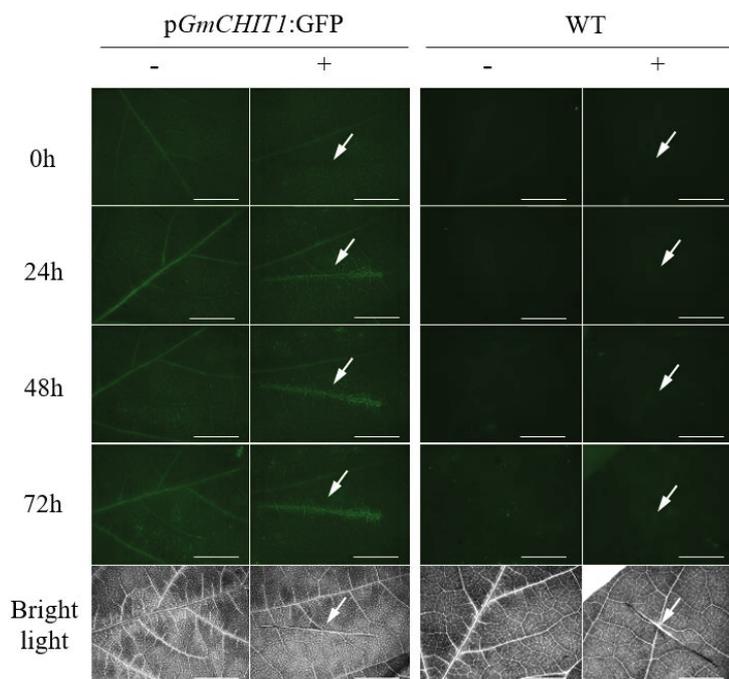


Figure 8: ***GmCHIT1* promoter response after wounding.** GFP fluorescence in wounded (+) or control (-) detached leaves from transgenic soybean (line 131 with the GFP fused to the *GmCHIT1* promoter) and WT plants. Scale bar represents 5 mm. Observations at 0, 24, 48 and 72 h after wounding with a dissection-scope (Leica Z16 APO) under GFP filter and bright light. Arrows show the wounded part.

## DISCUSSION

Many genes associated with disease resistance have been identified and proposed to develop transgenic plants to fight plant pathogens<sup>18,38,39</sup>. To drive the expression of these genes only as necessary, *i.e.*, during pathogen infection, the use of pathogen-inducible promoters is recommended. Such promoters have been isolated in several plants from genes associated with defence response<sup>40</sup>; for instance, the barley germin-like GER4 promoter, which controls the expression of a PR protein with superoxide dismutase activity highly induced by biotrophic and necrotrophic pathogens, was isolated<sup>21</sup>.

Nevertheless, the identification and characterization of such promoters in soybean is still limited<sup>23,41</sup>. This work presents the identification of a soybean chitinase gene (*GmCHIT1*) induced by *P. pachyrhizi*. The activity profile of the promoter of this gene was investigated in soybean plants by generating stable soybean events expressing the GFP under the control of p*GmCHIT1*.

Several studies revealed that genes associated with defence response, such as PR genes, are found to be induced during soybean rust contamination in both resistant and susceptible soybeans<sup>42</sup>. Among them, the *GmCHIT1* gene coding for a chitinase was reported as upregulated during early (24 hpi) and later (10 dpi) stages of *P. pachyrhizi* infection. We investigated the expression profile of this gene during the infectious process of *P. pachyrhizi* on soybean plants and confirmed that overexpression of this chitinase was detectable as early as 8 hpi and remained constant from 24 to 72 hpi, increasing drastically at 10 dpi. Considering the infectious development of *P. pachyrhizi*, appressorium formation and rust penetration in

plant tissues occur between six and twelve hours after urediniospore contamination<sup>43</sup>. Between 24 and 48 hpi, the fungus mainly forms haustoria and begins establishing secondary hyphae. During this phase, the pathogen development is low. However, the plant tissues are extensively colonized by the pathogen at 10 dpi<sup>43</sup>. Considering *GmCHIT1* expression, we can surmise that it could be induced through a plant signal during the appressorium formation and/or fungal penetration, and its expression could be proportional to the quantity of mycelia developing inside the plant tissues.

Heterologous systems are often used to study gene expression, but results produced in these experiments are limited because promoter regulation may depend on the genetic background of the plant species under investigation<sup>44-46</sup>. We therefore generated stable transgenic soybeans harbouring GFP under the control of the *GmCHIT1* promoter, thus using an authentic test system. This approach allowed us to highlight the local induction of the plant chitinase promoter around the fungal appressoria (Figure 5).

Mechanical injuries of plant tissues provide an entrance for pathogen invasion. Therefore, several wound-induced genes are also involved in plant defence pathways against invading fungi<sup>47</sup>. *P. pachyrhizi* penetrates directly the epidermal cells of the leaves rather than the stomata<sup>5</sup>. This action leads to the collapse of the epidermal cells. In this particular case of interaction, it is not surprising to observe that p*GmCHIT1*, a promoter activated by the pathogen, is also induced by wounding. The pattern of p*GmCHIT1* response to wounding is similar to the one observed by Hernandez-Garcia and Finer in wounded soybean plants harbouring the transcriptional fusion of the GFP and *GmERF3* promoter<sup>41</sup>. However, we cannot affirm that p*GmCHIT1* induction by wounding is the result of signalling associated solely with the tissue injury, the rust infection or both.

A majority of chitinase promoters have been shown to be induced by different pathogens and other abiotic stresses. For instance, the *BjChp* chitinase promoter of *Brassica juncea* has been reported to be induced by *Alternaria brassicae*, jasmonic acid and wounding in *A. thaliana*<sup>48</sup>. *BjChp* promoter activity was observed surrounding the necrotic lesions at 48 hpi. Another chitinase promoter of bean (*Phaseolus vulgaris* *PvChi4* promoter) has been reported to be expressed in lateral roots and reproductive organs of non-stressed *A. thaliana* plants<sup>49</sup>. This promoter was also induced by heat treatment and UV light. Additionally, the promoter of the chitinase AtEP3, the closest *A. thaliana* orthologue of *GmCHIT1*, was shown to be early induced by *Xanthomonas campestris* (at 1, 6 and 24 hpi) but downregulated by wounding<sup>50,51</sup>. These results indicated similar and different regulation activity among chitinase promoters.

Transcriptional regulation of defence genes under biotic stress is regulated by many *cis*-elements localized in the promoter<sup>19</sup>. Among them, GCC-box and W-boxes have been shown to be inducible by pathogens and wounding<sup>19</sup>. In the *ChiIV3* chitinase promoter of pepper, one W-box located in the -712/-459 bp region was described as essential to trigger the induction after *Phytophthora capsici* contamination<sup>52</sup>. W-box refers to the binding site of WRKY transcription factors<sup>32</sup>, and in soybean, these regulators have been shown to be implicated in the response to *P. pachyrhizi*<sup>53</sup>. In the *GmCHIT1* promoter, 21 W-boxes have been identified. In addition, 10 GT1-boxes and 5 MYB recognition elements have also been found. It has been demonstrated that GT1-boxes are responsible for the induction of defence genes by pathogen and salt stress. In the soybean promoter of the calmodulin *SCaM-4*, a GT1 *cis*-element was identified as essential for induction by *P. syringae* pv *tomato* and NaCl in *A. thaliana*<sup>54</sup>. MYB recognition elements were found in defence gene promoters and could be implicated in response

to abiotic stress and hormone treatment <sup>55</sup>. Finally, two auxin and two gibberellic acid responsive elements have been found in the *GmCHIT1* promoter. These observations suggest that *pGmCHIT1* could be potentially activated by these hormones. It would be interesting to investigate whether the *cis*-regulating elements found in the *GmCHIT1* promoter are essential and sufficient to trigger a response to *P. pachyrhizi*.

Fungal infection can induce different plant hormone pathways depending on the lifestyle of the pathogen. Usually, salicylate signalling is implicated in defence against biotrophic fungi and jasmonate together with ethylene participate in the defence against necrotrophic fungi <sup>56</sup>. However, a study of non-host interaction between *P. pachyrhizi* and *A. thaliana* has revealed that the pathogen activates marker genes of necrotrophic infection <sup>57</sup>. It has been suggested that the fungus would mimic a necrotrophic behaviour at the initial stage of infection to promote its development inside the host tissues <sup>58</sup>. In this context, one would expect *P. pachyrhizi* development to induce the jasmonic acid or ethylene pathway at early time-points after contamination and salicylic acid-related genes at later times. However, expression data during the early and late stages of *P. pachyrhizi* development did not reveal clear evidence of activation of either the salicylate or ethylene pathway <sup>29,42,58</sup>. Nevertheless, it was surprising to observe that the *GmCHIT1* promoter was not induced by any hormonal treatments assessed in our study. Indeed, several PR proteins have been shown to be activated by plant hormones <sup>59</sup>. For instance, a chitinase from rice has been reported to be induced by jasmonic acid and ethylene 48 h post-treatment <sup>60</sup>. However, unlike in Mazarei *et al.* <sup>61</sup>, *GmPRI* was not induced after our salicylic acid treatment and only a slight induction of *GmPR3* was observed. It is unclear at this stage whether the results reflect a lack of efficacy of salicylic acid treatment or an insensitivity of *pGmCHIT1* to this hormone.

Basal *GmCHIT1* promoter activity in non-contaminated soybean tissues was also investigated. We observed that *pGmCHIT1* was expressed in the veins of fully developed leaves and in roots but not in young leaves and flowers. Roots are permanently exposed to soil pathogens that can penetrate the tissues because of micro-wounds and the absence of lignified barriers <sup>62</sup>. This basal expression level in different soybean tissues/organs together with the induction under rust attack might reflect the potential roles of this chitinase in physiological processes of growth and development as much as in pathogen protection.

## CONCLUSIONS

Promoters are the primary regulators of gene expression at the transcriptional level and are considered as key elements to control transgenes in transgenic organisms. We identified *pGmCHIT1*, a promoter of soybean chitinase gene expressed during the early stages of leaf infection by *P. pachyrhizi*. To our knowledge, *pGmCHIT1* is the first soybean promoter reported as locally activated by *P. pachyrhizi* on plant tissue. Its characteristics suggest that it can be considered as a candidate of choice for driving defence genes in genetically engineered soybean.

## METHODS

### Construction of the transformation vectors

The GFP reporter gene<sup>63</sup> was amplified by PCR with primers gfp-F/gfp-R (see additional file 8: Table S4) and cloned downstream of the CsVMV promoter from Cassava Vein Mosaic Virus<sup>64</sup>. The *PDF1.2* promoter from *Arabidopsis thaliana*<sup>20</sup> was amplified by PCR using primers pdf1.2-F/ pdf1.2-R (see additional file 6: Table S3) and cloned to drive the expression of the GFP-encoding sequence. Upstream of the *Glyma.11G124500* gene-encoding sequence (based on *G. max* genome sequence from <https://phytozome.jgi.doe.gov/pz/portal.html>), a 3454 bp segment considered as part of the *GmCHIT1* promoter was synthesized by Eurofins genomic (Germany). The promoter was then cloned to drive the expression of the GFP-encoding gene. Each GFP construct was transferred to *A. tumefaciens* strain LBA4404. In all vectors, the HPPD (hydroxyphenylpyruvate dioxygenase) gene driven by the 35S promoter was used as a selectable marker for soybean transformation<sup>65</sup>.

### Soybean cultivation

Seeds of soybean cultivar Thorne, susceptible to *P. pachyrhizi*, were sown in pots containing SteckMedium substrate (Klasmann-Deilmann GmbH, Germany) for germination. After 3 weeks, the plants were transferred into larger pots for development and eventually seed production. Greenhouse conditions were as follows: temperature of 24 °C day/22 °C night with a photoperiod of 16 h of day under a light intensity of 270  $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  and 70% relative humidity.

### Soybean transformation

Seeds were surface sterilized for 24 h in a desiccator by chlorine gas generated with a mixture of 150 ml Domestos containing 4.5% NaClO w/w (Unilever) and 5 ml of HCl (37%). Sterile seeds were then hydrated overnight in sterile deionized water. Cotyledons of germinated seeds were dissected by removing the seed coat and by splitting the seeds into 2 halves using a scalpel blade. The half-seeds were immersed for 30 min in 10% W/V Gamborg's medium (Gamborg *et al.*, 1968) containing 30 g/l sucrose, 7.4  $\mu\text{M}$  BAP (6-benzylaminopurine), 0.7  $\mu\text{M}$  GA3 (gibberellic acid A3), 3.3 mM cysteine, 1 mM dithiothreitol, 200  $\mu\text{M}$  acetosyringone, 20 mM MES, pH 5.4 and the bacterium *Agrobacterium tumefaciens* at a final OD<sub>600nm</sub> of 0.8. Next, cotyledons were transferred to Petri dishes, adaxial side down, onto 3 layers of Whatman® paper pre-soaked with 10 ml of Gamborg's medium. Plates were transferred to a tissue culture room for 5 days at 24°C, 16 h light (180  $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) and 75% relative humidity. Shoots were induced by transferring the cotyledons to full-strength Gamborg's medium containing 30 g/L sucrose, 7.4  $\mu\text{M}$  BAP, 3 mM MES pH 5.6 and 8 g/l noble agar. Antibiotics ticarcillin (50 mg/l), cefotaxime (50 mg/l), vancomycin (50 mg/l) and the herbicide Tembotrione™ (0.2 mg/l) used as selectable marker were added after autoclaving. After one month on the shoot induction medium, white shoots were removed and cotyledons were transferred on a shoot elongation medium containing Murashige & Skoog (MS) salts<sup>66</sup>, 3.2 g/l Gamborg's vitamins, 30 g/l sucrose, 100 mg/l pyroglutamic acid, 50 mg/l asparagine, 0.28  $\mu\text{M}$  zeatin riboside, 0.57  $\mu\text{M}$  indol-3-acetic acid, 14.8  $\mu\text{M}$  GA3, 3 mM MES, pH 5.6 and 8 g/l noble agar. Antibiotics and the herbicide were kept at the same concentrations previously described. After one month, elongated shoots were cut and transferred to a rooting medium consisting of half-strength MS salts, half-strength B5 vitamins, 15 g/l sucrose, and 8 g/l noble agar. The same antibiotics as previously described were added after autoclaving, but the selectable marker was omitted.

When roots were sufficiently developed, the shoots were individually transplanted to a greenhouse and cultivated using the conditions previously described.

### **Characterization of transgenic plants**

Regenerated T0 events were confirmed for the presence of the selectable marker gene with an HPPD lateral flow test (AMAR Immunodiagnostics) using the experimental instructions recommended by the provider. To pick up T1 HPPD/GFP-positive events, germinated seeds were watered with an 8‰ solution of the herbicide Isoxaflutole™ to eliminate null segregant plants. Plants showing no herbicide symptoms were subsequently tested for GFP fluorescence and used for further analysis. Homozygous single-locus plants were selected either in T1 or T2 segregating generations by ddPCR analysis. T1 or T2 plants were used depending on the availability of the material.

### **Fungal contamination of soybean plants**

A dehydrated stock of spores of *P. pachyrhizi* stored in liquid nitrogen (isolate MG2006, Mato Grosso, Brazil 2006) was used as a routine source of inoculum. Twenty-four hours before plant contamination, cryo-tubes were opened and placed in a controlled growth chamber (20°C, dark, 70% relative humidity) to slowly rehydrate the spores. The spores were finally suspended in sterilized water containing 0.01% Tween 20 to reach a final concentration of 100,000 spores/ml. Three-week-old soybean plants were sprayed with the spores until run-off and incubated in a growth chamber (temperature 24°C, dark, 100% relative humidity) for 24 h before being transferred to a developing chamber (temperature of 24°C, 16 h light/8 h night, light intensity 15  $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  and 80% relative humidity). All experiments were conducted according to the recommendations of the French biosafety agency (Haut Conseil des Biotechnologies).

### **Treatment of detached soybean leaves**

First and second trifoliolate leaves of 6-week-old plants were excised and transferred to layers of Whatman® paper wetted with 6 ml of sterile distilled water. Leaf petioles were wrapped with water-soaked cotton to increase organ survival. Different hormone treatments were conducted by spraying leaves with either 20 mM of ACC (ethylene precursor) or 2.5 mM solution of salicylic acid (SA) in sterile water or 0.25 mM of coronatine (methyl jasmonate analogue) in 1% EC premix solution (phenyl sulfonate 5%, emulsogen EL360 7%, isophorone 40% and methyloleate 48%). Sterile distilled water was used as mock for ACC and SA treatments, and 1% EC premix was used as mock for coronatine spray. Leaf wounding was realized with a sterile scalpel blade. After the different treatments, the leaves were incubated in the same growth chamber used for soybean transformation. Macroscopic observations and fluorescence intensity measurement were performed at 24, 48 and 72 h post-treatment.

### **Expression profiling by quantitative PCR analysis**

Samples were composed of four foliar discs from leaves of a soybean plant, and three independent biological replicates were performed. Total RNA was extracted using the RNeasy® Plant Mini Kit (Qiagen, Netherlands) and purified with the TURBO DNA-free™ Kit (Invitrogen, Carlsbad, CA). DNA-free total RNA (1  $\mu\text{g}$ ) was used to synthesize cDNA with the ThermoScript™ RT-PCR System kit (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. For RT-qPCR, 0.02  $\mu\text{g}$  of cDNA was used in a 20  $\mu\text{l}$  reaction

containing 10 µl of SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, US), 6 µM of forward and reverse primers and 3 µl of RNase-free water. RT-qPCR was performed using the LightCycler® 480. The thermocycling conditions were followed as recommended by the supplier. The expression of the chitinase gene was determined after soybean rust inoculation by using a specific primer (see additional file 6: Table S3). The genes coding for actin (GenBank: NM\_001289231.2) and a hypothetical protein (GenBank: BE330043)<sup>65</sup> (primer sequence in additional file 6: Table S3) were used as endogenous reference genes for normalization<sup>67</sup> using the Ct value method. Specific primers of *GmPR1* (GenBank: BU5773813), *GmPR2* (GenBank: M37753) and *GmPR3* (GenBank: AF202731) were used to determine the expression of those *PR* genes after salicylic acid treatment. In this case, the genes coding for actin and an elongation factor (GenBank: NM\_001249608.2) were used for normalization (see additional file 6: Table S3) with the Ct value method.

### Western blot analysis

Leaf samples from wild-type (WT) plants and plants from lines 129, 131 and 133 were harvested 72 h after *P. pachyrhizi* contamination or mock treatment. Proteins were extracted from four foliar discs of the same soybean plant with 250 µl of extraction buffer (Tris-Hcl 100 mM, NaCl 100 mM, DDT 0.04%) and placed on ice for 10 min before centrifugation at 4°C for 10 min. The protein concentration was determined with the Bradford method using the Bio-Rad Protein assay dye reagent solution. For denaturation, 1 volume of Laemmli buffer (Bio-Rad, US) was added to 1 volume of extracted protein (30 µg). The mixture was kept for 5 min at 95°C and 5 min on ice before loading on a TGX 4-20% Strainfree (Bio-Rad US) gel immersed in TGS 1X buffer. After migration, separated proteins were transferred onto a membrane by using Trans-Blot® Turbo™ Midi Nitrocellulose Transfer Packs (Bio-Rad) and the TransBlot Turbo device (Bio-Rad, US). Membrane blocking and incubation with the antibodies were performed as suggested by the provider. GFP antibodies (Sigma) and Immun-Star Goat Anti-Rabbit (GAR)-HRP Conjugate antibody were used. Antibody revelation was realized with the Clarity™ Western ECL (Bio-Rad, US) kit following the supplier's instructions. Finally, the ChemiDoc™ Touch camera (Bio-Rad, US) was used to record the results.

### Visualization of GFP expression

GFP fluorescence was analysed with a Leica Z16 APO A dissection scope equipped with a GFP filter. For the detection of fluorescence after rust inoculation, the parameters were set as follows: camera lens 1 x, magnification 115 x, gain 2 and exposure time 500 ms. For detection of the GFP fluorescence in the different soybean tissues without infection, the camera lens was set at camera lens 0.5 x, magnification at 6.95 x for roots and young trifoliolate leaves and 15 x for flowers, gain 3, exposure time 500 ms. For hormonal treatments and wounding, the following parameters were used: camera lens 1 x, magnification 6.95 x, gain 3 and exposure time 1 s. Fluorescence intensity measurement was performed using MetaMorph software *via* greyscale value.

### Confocal microscopy

Leaf samples of soybean line 131 expressing the transcriptomic fusion p*GmCHIT1*:GFP were harvested 24 h post-contamination. The samples were first stained in an aqueous calcofluor white solution (0.01 mg/ml) for 5 min before being washed 3 times in water for 5 min. Samples

were mounted in water under slides (VWR® microscope slides: ground edges 45°, 76 x 26 mm) and cover glass (VWR® cover glass: 22 x 32 mm). Observations were conducted with a ZEISS LSM 800 microscope using the 10x objective. To visualize GFP fluorescence, a 487 nm wavelength laser was used for excitation and light emission was captured at 560 nm. For the imaging of calcofluor fluorescence, light excitation was set at a wavelength of 400 nm and emission was captured at 487 nm.

## ADDITIONAL FILES

**Additional file 1: Table S1.** List of the soybean genes upregulated after *P. pachyrhizi* contamination. Soybean genes identified in Tremblay *et al.*, 2010, and Tremblay *et al.*, 2011. Genes were re-annotated with the last soybean genome notation available: Glycine max 275 William 82 (from <https://genome.jgi.doe.gov/portal/soybean/soybean.home.html>). The genes were ranked according to their relative expression level compared to the mock inoculation.

Gene	Gene_Description	Function_2010	Rank 2010	Rank 2011
Glyma.02G145200	E3 ubiquitin ligase involved in syntaxin degradation	Cell Growth & Division	213	1297
Glyma.12G022500	E3 ubiquitin ligase involved in syntaxin degradation	Cell Growth & Division	199	542
Glyma.10G275200	Uncharacterized conserved protein	Cell Growth & Division	260	989
Glyma.10G212900	Translation elongation factor EF-1 alpha/Tu	Cell Growth & Division	141	53
Glyma.08G358300	26S proteasome regulatory complex, subunit PSMD10	Cell Structure	467	1930
Glyma.12G053900	Beta-glucosidase, lactase phlorizinhydrolase, and related proteins	Cell Structure	259	837
Glyma.14G217100	Histones H3 and H4	Cell Structure	492	3113
Glyma.18G288600	Phospholipase D1	Cell Structure	490	906
Glyma.02G093500	Phospholipase D1	Cell Structure	258	2633
Glyma.17G008000	Predicted membrane protein, contains DoH and Cytochrome b-561/ferric reductase transmembrane domains	Cell Structure	22	186
Glyma.07G266300	Predicted membrane protein, contains DoH and Cytochrome b-561/ferric reductase transmembrane domains	Cell Structure	294	1663
Glyma.13G091000	Tetraspanin family integral membrane protein	Cell Structure	164	1693
Glyma.17G159300	Uncharacterized membrane protein	Cell Structure	562	4352
Glyma.14G004300	Predicted NUDIX hydrolase FGF-2 and related proteins	Cell Structure	15	61
Glyma.02G126800	Protein transporter of the TRAM (translocating chain-associating membrane) superfamily	Cell Structure	401	189
Glyma.02G107500	Membrane associated zinc finger	Cell Structure	313	4512
Glyma.19G054700	Leucine rich repeat protein	Disease & Defence	266	134
Glyma.05G082200	Leucine rich repeat protein	Disease & Defence	379	466
Glyma.17G179200	Leucine rich repeat protein	Disease & Defence	595	439

Glyma.05G055000	Copper chaperone for superoxide dismutase	Disease & Defence	198	2136
Glyma.15G062400	Defence-related protein containing SCP domain	Disease & Defence	14	1
Glyma.15G062500	Defence-related protein containing SCP domain	Disease & Defence	11	22
Glyma.10G152200	Ferric reductase, NADH/NADPH oxidase and related proteins	Disease & Defence	288	1302
Glyma.19G233900	Ferric reductase, NADH/NADPH oxidase and related proteins	Disease & Defence	479	2405
Glyma.04G254100	Galactosyltransferases	Disease & Defence	422	1897
Glyma.13G346700	Predicted chitinase	Disease & Defence	2	5
Glyma.11G124500	Predicted chitinase	Disease & Defence	4	40
Glyma.20G150200	dioxygenase	Metabolism	545	1002
Glyma.10G244100	dioxygenase	Metabolism	542	1568
Glyma.04G232200	AAA+type ATPase	Energy	183	3779
Glyma.19G018600	AAA+type ATPase	Energy	145	157
Glyma.07G052400	Cytochrome b5	Energy	454	1188
Glyma.16G019900	D-arabinono-1,4-lactone oxidase	Energy	466	34
Glyma.06G107200	Glutaredoxin and related proteins	Energy	189	493
Glyma.01G210400	Lactate dehydrogenase	Energy	19	556
Glyma.05G058100	Lactate dehydrogenase	Energy	443	3469
Glyma.17G128000	Malate synthase	Energy	345	52
Glyma.11G179300	NADH-dehydrogenase (ubiquinone)	Energy	90	8
Glyma.06G266700	Thioredoxin	Energy	123	2951
Glyma.08G285900	Thioredoxin, nucleoredoxin and related proteins	Energy	300	362
Glyma.04G021000	Thioredoxin, nucleoredoxin and related proteins	Energy	565	1096
Glyma.08G277000	Transketolase	Energy	84	214
Glyma.08G162200	Uncharacterized high-glucose-regulated protein	Energy	548	3069
Glyma.03G246700	Voltage-gated shaker-like K channel, subunit beta/KCNAB	Energy	575	1710
Glyma.17G076600	SNAP-25 (synaptosome-associated protein) component of SNARE complex	Intracellular Traffic	201	754
Glyma.05G023100	SNAP-25 (synaptosome-associated protein) component of SNARE complex	Intracellular Traffic	257	2916
Glyma.13G180100	AAA+type ATPase	Intracellular Traffic	444	3780
Glyma.04G060700	Endosomal membrane proteins, EMP70	Intracellular Traffic	588	1952
Glyma.05G047800	Exocyst component protein and related proteins	Intracellular Traffic	468	1163
Glyma.03G174300	Exocyst component protein and related proteins	Intracellular Traffic	321	280
Glyma.15G013900	G-protein beta subunit	Intracellular Traffic	61	621
Glyma.17G146100	GTPase Rab1/YPT1, small G protein superfamily, and related GTP-binding proteins	Intracellular Traffic	473	5641
Glyma.08G239900	Phosphatidylinositol transfer protein SEC14 and related proteins	Intracellular Traffic	346	3260
Glyma.18G013100	Predicted E3 ubiquitin ligase	Intracellular Traffic	517	743
Glyma.19G051500	Predicted E3 ubiquitin ligase	Intracellular Traffic	442	5589
Glyma.15G069100	Secretory carrier membrane protein	Intracellular Traffic	104	5353

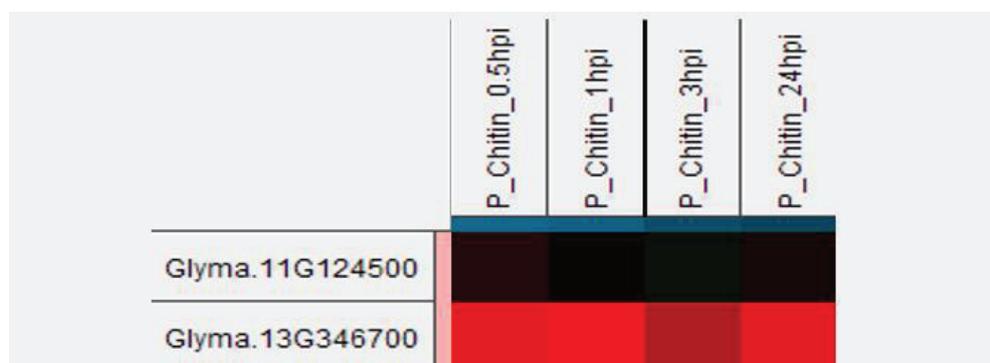
Glyma.02G195400	SNARE protein Syntaxin 1 and related proteins	Intracellular Traffic	166	755
Glyma.02G195300	SNARE protein Syntaxin 1 and related proteins	Intracellular Traffic	102	151
Glyma.13G136600	SNARE protein YKT6, synaptobrevin/VAMP superfamily	Intracellular Traffic	581	5188
Glyma.08G254500	6-phosphogluconate dehydrogenase	Metabolism	320	812
Glyma.10G200700	Acetylglucosaminyltransferase EXT2/exostosin 2	Metabolism	428	576
Glyma.14G042000	Alcohol dehydrogenase, class III	Metabolism	458	1048
Glyma.02G034000	Aldehyde dehydrogenase	Metabolism	82	727
Glyma.05G231800	Aldehyde dehydrogenase	Metabolism	350	1225
Glyma.18G285800	Aldo/keto reductase family proteins	Metabolism	417	816
Glyma.02G307300	Aldo/keto reductase family proteins	Metabolism	34	2205
Glyma.03G148300	Arylacetamide deacetylase	Metabolism	299	1555
Glyma.01G239600	Arylacetamide deacetylase	Metabolism	48	5047
Glyma.18G061100	Asparagine synthase (glutamine-hydrolysing)	Metabolism	36	60
Glyma.12G005100	Beta-fructofuranosidase (invertase)	Metabolism	160	1255
Glyma.08G028200	Choline phosphate cytidyltransferase/Predicted CDP-ethanolamine synthase	Metabolism	606	5615
Glyma.11G091400	Copper chaperone	Metabolism	148	2
Glyma.10G172700	Cystathionine beta-lyases/cystathionine gamma-synthases	Metabolism	88	204
Glyma.08G140600	Cytochrome P450 CYP2 subfamily	Metabolism	163	33
Glyma.13G285300	Cytochrome P450 CYP2 subfamily	Metabolism	165	143
Glyma.11G062500	Cytochrome P450 CYP2 subfamily	Metabolism	85	254
Glyma.13G173500	Cytochrome P450 CYP2 subfamily	Metabolism	170	287
Glyma.07G089800	Cytochrome P450 CYP2 subfamily	Metabolism	175	376
Glyma.15G156100	Cytochrome P450 CYP2 subfamily	Metabolism	63	378
Glyma.13G068800	Cytochrome P450 CYP2 subfamily	Metabolism	211	608
Glyma.03G143700	Cytochrome P450 CYP2 subfamily	Metabolism	597	1282
Glyma.07G202300	Cytochrome P450 CYP2 subfamily	Metabolism	274	1570
Glyma.18G080400	Cytochrome P450 CYP2 subfamily	Metabolism	452	2347
Glyma.03G031000	Cytochrome P450 CYP2 subfamily	Metabolism	614	3714
Glyma.13G262000	Cytochrome P450 CYP4/CYP19/CYP26 subfamilies	Metabolism	327	455
Glyma.04G035000	Cytochrome P450 CYP4/CYP19/CYP26 subfamilies	Metabolism	485	2352
Glyma.11G215700	Diadenosine and diphosphoinositol polyphosphate phosphohydrolase	Metabolism	240	1951
Glyma.19G050100	GDP-mannose pyrophosphorylase	Metabolism	456	1308
Glyma.16G063200	Glucose-6-phosphate 1-dehydrogenase	Metabolism	393	1600
Glyma.11G213000	Glutamate decarboxylase/sphingosine phosphate lyase	Metabolism	75	344
Glyma.13G233300	Glutamate-gated kainate-type ion channel receptor subunit GluR5 and related subunits	Metabolism	267	615
Glyma.11G215500	Glutamine synthetase	Metabolism	157	152
Glyma.18G041100	Glutamine synthetase	Metabolism	101	436
Glyma.12G207400	Glutaredoxin and related proteins	Metabolism	78	1601
Glyma.10G139400	Haloacid dehalogenase-like hydrolase	Metabolism	448	3704

Glyma.07G048900	Hydroxyindole-O-methyltransferase and related SAM-dependent methyltransferases	Metabolism	195	10
Glyma.09G281900	Hydroxyindole-O-methyltransferase and related SAM-dependent methyltransferases	Metabolism	619	67
Glyma.01G225200	Long-chain acyl-CoA synthetases (AMP-forming)	Metabolism	488	717
Glyma.17G044300	Lysine-ketoglutarate reductase/saccharopine dehydrogenase	Metabolism	569	182
Glyma.10G291400	Lysophospholipase	Metabolism	344	583
Glyma.13G354900	NADP+dependent malic enzyme	Metabolism	463	3131
Glyma.19G011700	Peroxidase/oxygenase	Metabolism	27	167
Glyma.02G309900	Phosphorylcholine transferase/cholinephosphate cytidylyltransferase	Metabolism	533	1607
Glyma.13G210000	Predicted hydrolase related to diene lactone hydrolase	Metabolism	121	20
Glyma.19G099400	Predicted hydrolase/acyltransferase (alpha/beta hydrolase superfamily)	Metabolism	285	2048
Glyma.01G205900	Predicted lipase	Metabolism	395	4099
Glyma.02G148400	Predicted PhzC/PhzF-type epimerase	Metabolism	210	97
Glyma.16G042000	Reductases with broad range of substrate specificities	Metabolism	203	446
Glyma.17G067800	Trehalose-6-phosphate synthase component TPS1 and related subunits	Metabolism	108	284
Glyma.09G030300	UDP-glucose 4-epimerase/UDP-sulfoquinovose synthase	Metabolism	370	3596
Glyma.08G244800	UDP-glucuronosyl and UDP-glucosyl transferase	Metabolism	380	36
Glyma.08G244700	UDP-glucuronosyl and UDP-glucosyl transferase	Metabolism	357	64
Glyma.02G104300	UDP-glucuronosyl and UDP-glucosyl transferase	Metabolism	505	137
Glyma.11G000500	UDP-glucuronosyl and UDP-glucosyl transferase	Metabolism	335	768
Glyma.19G243300	Uncharacterized conserved protein	Metabolism	559	3696
Glyma.06G135100	Predicted RNA-binding protein SEB4 (RRM superfamily)	Post-Transcription	514	400
Glyma.15G154000	Cullins	Protein Destination & Storage	451	2137
Glyma.04G049900	Asparaginyl peptidases	Protein Destination & Storage	593	1011
Glyma.12G179800	Aspartyl protease	Protein Destination & Storage	177	831
Glyma.01G163000	Aspartyl protease	Protein Destination & Storage	152	1018
Glyma.05G064700	Bax-mediated apoptosis inhibitor TEGT/BI-1	Protein Destination & Storage	475	2019
Glyma.15G177800	Cysteine proteinase Cathepsin L	Protein Destination & Storage	360	107
Glyma.07G140000	Glutathione S-transferase	Protein Destination & Storage	205	163
Glyma.16G130700	Hydrolytic enzymes of the alpha/beta hydrolase fold	Protein Destination & Storage	394	1588
Glyma.06G120600	Leucine rich repeat proteins, some proteins contain F-box	Protein Destination & Storage	520	352
Glyma.17G049600	Leucine rich repeat proteins, some proteins contain F-box	Protein Destination & Storage	605	884
Glyma.06G116300	Mitochondrial inner membrane protease, subunit IMP1	Protein Destination & Storage	303	1967
Glyma.11G018500	Mitochondrial solute carrier protein	Protein Destination & Storage	430	3999
Glyma.13G289600	Molecular chaperone (DnaJ superfamily)	Protein Destination & Storage	287	293
Glyma.04G190800	Peptide methionine sulfoxide reductase	Protein Destination & Storage	132	71

Glyma.06G317800	Predicted membrane protein	Protein Destination & Storage	498	24
Glyma.15G156200	Serine carboxypeptidases (lysosomal cathepsin A)	Protein Destination & Storage	265	26
Glyma.13G117700	Ubiquitin and ubiquitin-like proteins	Protein Destination & Storage	129	285
Glyma.13G117900	Ubiquitin and ubiquitin-like proteins	Protein Destination & Storage	531	2159
Glyma.15G091400	Ubiquitin-like protein	Protein Destination & Storage	566	724
Glyma.04G238800	Ubiquitin-protein ligase	Protein Destination & Storage	438	5484
Glyma.07G060900	Translation initiation factor 1A (eIF-1A)	Protein Synthesis	564	1616
Glyma.02G236500	Cytochrome P450 CYP2 subfamily	Secondary Metabolism	497	5406
Glyma.05G147000	O-methyltransferase	Secondary Metabolism	46	9
Glyma.02G239500	Acyl-CoA synthetase	Secondary Metabolism	91	115
Glyma.06G202300	Cytochrome P450 CYP2 subfamily	Secondary Metabolism	301	3738
Glyma.09G269500	Flavonol reductase/cinnamoyl-CoA reductase	Secondary Metabolism	107	161
Glyma.09G269600	Flavonol reductase/cinnamoyl-CoA reductase	Secondary Metabolism	83	51
Glyma.02G158700	Flavonol reductase/cinnamoyl-CoA reductase	Secondary Metabolism	383	90
Glyma.20G213700	Hydroxyindole-O-methyltransferase and related SAM-dependent methyltransferases	Secondary Metabolism	196	87
Glyma.07G168500	Iron/ascorbate family oxidoreductases	Secondary Metabolism	232	196
Glyma.15G223900	NADH:flavin oxidoreductase/12-oxophytodienoate reductase	Secondary Metabolism	122	314
Glyma.03G181600	Phenylalanine and histidine ammonia-lyase	Secondary Metabolism	126	2623
Glyma.12G059100	Reductases with broad range of substrate specificities	Secondary Metabolism	47	218
Glyma.14G004500	Predicted unusual protein kinase	Signal Transduction	599	923
Glyma.05G082400	Apoptotic ATPase	Signal Transduction	280	75
Glyma.03G138000	Ca <sup>2+</sup> dependent protein kinase, EF-Hand protein superfamily	Signal Transduction	297	4622
Glyma.19G257800	Ca <sup>2+</sup> binding protein (centrin/caltractin), EF-Hand superfamily protein	Signal Transduction	339	437
Glyma.14G081300	Ca <sup>2+</sup> independent phospholipase A2	signal Transduction	66	117
Glyma.04G245000	Calmodulin and related proteins (EF-Hand superfamily)	Signal Transduction	25	89
Glyma.02G245700	Calmodulin and related proteins (EF-Hand superfamily)	Signal Transduction	131	711
Glyma.04G194800	Calmodulin and related proteins (EF-Hand superfamily)	Signal Transduction	221	1264
Glyma.02G002100	Calmodulin and related proteins (EF-Hand superfamily)	Signal Transduction	188	1266
Glyma.19G244300	Calmodulin and related proteins (EF-Hand superfamily)	Signal Transduction	191	2286
Glyma.16G142500	Calmodulin and related proteins (EF-Hand superfamily)	Signal Transduction	229	3426
Glyma.15G212400	Cell cycle control protein	Signal Transduction	119	786
Glyma.02G257600	Dual-specificity tyrosine-phosphorylation regulated kinase	Signal Transduction	277	775
Glyma.18G298300	Multifunctional chaperone (14-3-3 family)	Signal Transduction	372	5003
Glyma.17G153800	Serine/threonine phosphatase	Signal Transduction	161	2794
Glyma.12G140200	Serine/threonine protein kinase	Signal Transduction	515	15
Glyma.09G014900	Serine/threonine protein kinase	Signal Transduction	38	191
Glyma.20G138500	Serine/threonine protein kinase	Signal Transduction	42	354

Glyma.13G073900	Serine/threonine protein kinase	Signal Transduction	364	411
Glyma.18G214000	Serine/threonine protein kinase	Signal Transduction	409	939
Glyma.18G275700	Serine/threonine protein kinase	Signal Transduction	381	2800
Glyma.18G216800	Serine/threonine protein kinase	Signal Transduction	367	136
Glyma.04G230500	Serine/threonine protein kinase	Signal Transduction	459	150
Glyma.10G109200	Serine/threonine protein kinase	Signal Transduction	304	331
Glyma.10G126700	Serine/threonine protein kinase	Signal Transduction	449	408
Glyma.20G140100	Serine/threonine protein kinase	Signal Transduction	219	417
Glyma.04G042400	Serine/threonine protein kinase	Signal Transduction	150	448
Glyma.20G054500	Serine/threonine protein kinase	Signal Transduction	307	753
Glyma.08G235900	Serine/threonine protein kinase	Signal Transduction	368	1434
Glyma.09G089700	Serine/threonine protein kinase	Signal Transduction	322	1826
Glyma.20G139700	Serine/threonine protein kinase	Signal Transduction	441	4831
Glyma.17G056900	Serine/threonine protein kinase	Signal Transduction	355	211
Glyma.20G138800	Serine/threonine protein kinase	Signal Transduction	374	932
Glyma.13G102200	Serine/threonine protein kinase	Signal Transduction	284	1459
Glyma.05G237100	Serine/threonine protein kinase	Signal Transduction	296	2831
Glyma.02G009100	Serine/threonine protein phosphatase	Signal Transduction	511	3557
Glyma.12G170100	SOK1 kinase belonging to the STE20/SPS1/GC kinase family	Signal Transduction	408	1017
Glyma.05G036600	Tyrosine kinase specific for activated (GTP-bound) p21cdc42Hs	Signal Transduction	590	2130
Glyma.07G142000	Uncharacterized conserved protein	Signal Transduction	521	3604
Glyma.14G016300	CCCH-type Zn-finger protein	Transcription	431	1274
Glyma.03G060200	HMG box-containing protein	Transcription	583	1173
Glyma.15G272400	Predicted E3 ubiquitin ligase	Transcription	519	4432
Glyma.11G246100	Predicted E3 ubiquitin ligase	Transcription	407	1380
Glyma.03G179300	Predicted E3 ubiquitin ligase	Transcription	331	3510
Glyma.05G178200	Uncharacterized conserved protein, contains IPT/TIG domain	Transcription	477	3055
Glyma.16G062600	Amino acid transporters	Transporter	109	140
Glyma.19G076800	Amino acid transporters	Transporter	76	298
Glyma.05G043100	Amino acid transporters	Transporter	315	4608
Glyma.10G132300	Ammonia permease	Transporter	72	223
Glyma.05G211600	Copper chaperone	Transporter	311	241
Glyma.07G065800	Copper chaperone	Transporter	579	733
Glyma.06G191300	Lipid exporter ABCA1 and related proteins, ABC superfamily	Transporter	114	312
Glyma.18G050400	Mitochondrial Fe2 transporter MMT1 and related transporters (cation diffusion facilitator superfamily)	Transporter	97	1023
Glyma.10G276700	Monocarboxylate transporter	Transporter	158	109
Glyma.20G112900	Monocarboxylate transporter	Transporter	604	798
Glyma.10G019000	Multidrug resistance-associated protein/mitoxantrone resistance protein, ABC superfamily	Transporter	286	101
Glyma.02G008000	Multidrug/pheromone exporter, ABC superfamily	Transporter	446	558
Glyma.09G056300	Plasma membrane H <sup>+</sup> -transporting ATPase	Transporter	212	184
Glyma.13G097900	Plasma membrane H <sup>+</sup> -transporting ATPase	Transporter	167	317
Glyma.14G134100	Plasma membrane H <sup>+</sup> -transporting ATPase	Transporter	423	788

Glyma.15G011900	Pleiotropic drug resistance proteins (PDR1-15), ABC superfamily	Transporter	233	12
Glyma.13G361900	Pleiotropic drug resistance proteins (PDR1-15), ABC superfamily	Transporter	26	79
Glyma.01G223600	Predicted K <sup>+</sup> antiporter	Transporter	332	1386
Glyma.13G131200	Predicted K <sup>+</sup> antiporter	Transporter	269	1889
Glyma.01G156200	Predicted membrane protein	Transporter	546	283
Glyma.01G238800	Predicted transporter (major facilitator superfamily)	Transporter	128	69
Glyma.08G035300	Predicted transporter (major facilitator superfamily)	Transporter	518	126
Glyma.19G020000	Prohibitins and stomatins of the PID superfamily	Transporter	283	291
Glyma.13G065000	Prohibitins and stomatins of the PID superfamily	Transporter	171	1582
Glyma.02G021400	Prohibitins and stomatins of the PID superfamily	Transporter	406	2729
Glyma.02G129500	P-type ATPase	Transporter	241	925

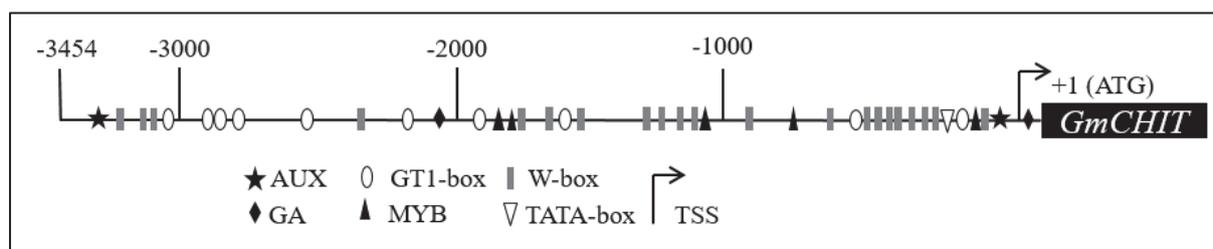


**Additional file 2: Figure S1.** Gene expression of *Glyma.13G346700* and *Glyma.11G124500* after chitinheptaose (DP7) treatment. Relative expression compared to that in the control plants (untreated) at 0.5, 1, 3, and 24 h post-treatment. Black: no change in expression with the DP7; red: upregulation with the DP7. Unpublished data.

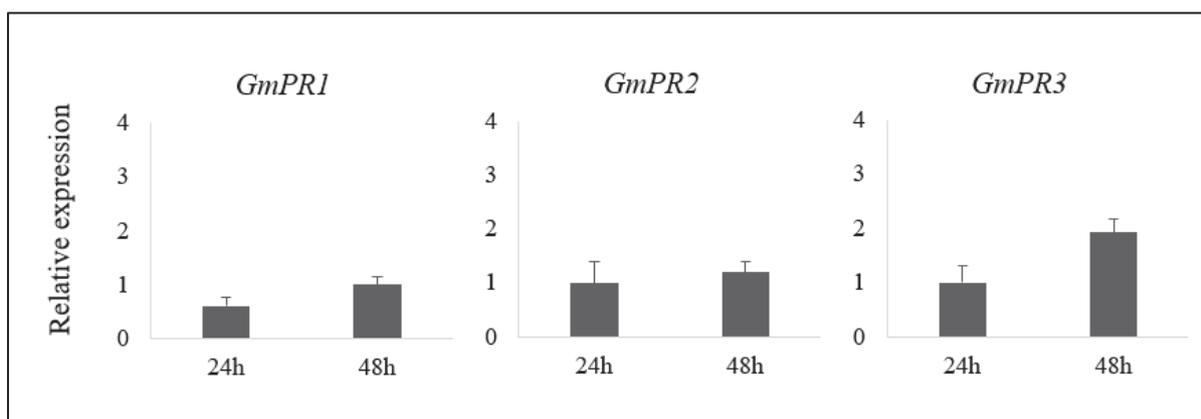
**Additional file 3: Table S2.** List of the cis-regulatory elements related to pathogen infection identified in the *GmCHIT1* promoter.

Factor or Site Name	Location	Strain	Signal Sequence	Description
ARFAT	85	(+)	TGTCTC	AUX
AUXREPSIAA4	3278	(-)	KGTCCCAT	AUX
CAREOSREP1	19	(+)	CAACTC	GA
CAREOSREP1	2019	(-)	CAACTC	GA
GT1GMSCAM4	225	(-)	GAAAAA	pathogen salt induction
GT1GMSCAM4	566	(+)	GAAAAA	pathogen salt induction
GT1GMSCAM4	1644	(+)	GAAAAA	pathogen salt induction
GT1GMSCAM4	1922	(-)	GAAAAA	pathogen salt induction
GT1GMSCAM4	2331	(+)	GAAAAA	pathogen salt induction
GT1GMSCAM4	2533	(+)	GAAAAA	pathogen salt induction
GT1GMSCAM4	2769	(-)	GAAAAA	pathogen salt induction
GT1GMSCAM4	2860	(-)	GAAAAA	pathogen salt induction
GT1GMSCAM4	2878	(-)	GAAAAA	pathogen salt induction

GT1GMSCAM4	3031	(-)	GAAAAA	pathogen salt induction
MYBST1	122	(-)	GGATA	MYB TF (biotic/abiotic response)
MYBST1	798	(-)	GGATA	MYB TF (biotic/abiotic response)
MYBST1	1131	(-)	GGATA	MYB TF (biotic/abiotic response)
MYBST1	1819	(-)	GGATA	MYB TF (biotic/abiotic response)
MYBST1	1888	(-)	GGATA	MYB TF (biotic/abiotic response)
TATABOX2	332	(+)	TATAAAT	TATA box
WBBOXPCWRKY1	485	(+)	TTTGACY	W_box
WBBOXPCWRKY1	517	(+)	TTTGACY	W_box
WBBOXPCWRKY1	1276	(+)	TTTGACY	W_box
WBBOXPCWRKY1	3102	(-)	TTTGACY	W_box
WBBOXPCWRKY1	3233	(-)	TTTGACY	W_box
WBOXATNPR1	401	(-)	TTGAC	W_box
WBOXATNPR1	410	(+)	TTGAC	W_box
WBOXATNPR1	883	(-)	TTGAC	W_box
WBOXATNPR1	1672	(-)	TTGAC	W_box
WBOXHVISO1	117	(+)	TGACT	W_box
WBOXHVISO1	479	(-)	TGACT	W_box
WBOXHVISO1	504	(+)	TGACT	W_box
WBOXHVISO1	696	(+)	TGACT	W_box
WBOXHVISO1	1354	(+)	TGACT	W_box
WBOXHVISO1	3169	(-)	TGACT	W_box
WBOXNTERF3	1380	(-)	TGACY	W_box
WRKY71OS	480	(-)	TGAC	W_box
WRKY71OS	1175	(-)	TGAC	W_box
WRKY71OS	1589	(-)	TGAC	W_box
WRKY71OS	1802	(-)	TGAC	W_box
WRKY71OS	2385	(+)	TGAC	W_box



**Additional file 4: Figure S2.** Map of *GmCHIT1* promoter and potential cis-regulatory elements identified. AUX: auxin-responsive element; GA: gibberellic acid-responsive elements; MYB: MYB recognition elements; GT1-box: pathogen and NaCl-responsive elements; W-box: pathogen-responsive elements; TSS: transcription start site.



**Additional file 5: Figure S3.** *GmPR1* (GenBank: BU5773813), *GmPR2* (GenBank: M37753), and *GmPR3* (GenBank: AF202731) expression in line 131 (*pGmCHIT1:GFP*) after SA treatment. Transcript accumulation at 24 and 48 h compared to that in the mock-treated plants. The actin (GenBank: NM\_001289231.2) and an elongation factor (GenBank: NM\_001249608.2) encoding genes were used as references. Three independent biological replicates  $\pm$  standard deviations were performed.

**Additional file 6: Table S3.** Primers used for PCR and qPCR.

Amplified sequence	Primer name	Primer sequence	Amplicon length	Primer use	
GFP	gfp F	GAATATTCACGTGAATTCATGGTGAGCAAGGGCGAGGAGC	759bp	PCR for cloning	
	gfp R	ACCTGCAGGCCACAGCTGTGGTTACTGTACAGCTCGTCCATGCCG			
PDF1.2 promoter	pdf1.2 F	GTAATACGCGTCTTAAGACGCTGGGACAAGCTTTATATGCAG	1253bp		
	pdf1.2 R	GGACGTAACATGAATTCGATGATTACTATTTTG			
Actin	GmACTIN F	CGGTGGTCTATCTTGGCATC	142bp		quantitative PCR
	GmACTIN R	GTCTTTCGCTTCAATAACCCTA			
Hypothetical protein	GmUKN2 F*	GCCTGGATACCTGCTCAAG	79bp		
	GmUKN2 R*	ACCTCCTCCTCAAACCTCCTCG			
Chitinase	GmCHIT F	GAGATTAACGGTGCATCAGG	330bp		
	GmCHIT R	ATTAACACGAGCCTGAACAGTACT			
GmPR1	GmPR1 F**	AACTATGCTCCCCTGGCAACTATATTG	76bp		
	GmPR1 R**	TCTGAAGTGGTAGCTTCTACATCGAAACAA			
GmPR2	GmPR2 F***	GTCTCCTTCGGTGGTAGTG	104bp		
	GmPR2 R***	ACCCTCCTCCTGCTTTCTC			
GmPR3	GmPR3 F***	GCATTGGTCTGGATTG	115bp		
	GmPR3 R***	GGCTTGATGGCTTGTTC			
Elongation factor	GmELF F	GTTGAAAAGCCAGGGGACA	79bp		
	GmELF R	TCTTACCCCTGAGCGTGG			

\*from Hirschburger et al., 2015, \*\* from Mazarei et al., 2007, \*\*\* from Zhong et al., 2014.

#### ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable

#### CONSENT FOR PUBLICATION

Not applicable

#### AVAILABILITY OF DATA AND MATERIAL

All the data and material generated are BASF property.

#### COMPETING INTERESTS

All authors except NP and LG are inventors of the linked patent WO2018217474. SD, BP, SP, and CS are employees of Bayer Company.

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## AUTHORS' CONTRIBUTIONS

NP, BP, SD and LC conceived and designed the experiments. SP performed the gene expression analyses, SP and CS generated the phylogenetic tree, LG performed the soybean transformations and LC the remainder of the experiments. NP, BP, SD, CS, SP and LC analysed the data. NP, BP, SD and LC wrote the paper. All the authors have read and approved the manuscript.

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## E. DISCUSSION

Five candidate promoters potentially suitable to control the barnase gene expression were identified. In order to precise which one could be used in a cell death technology, we generated stable transgenic soybeans expressing a promoter:GFP cassette. The objective of this first step was to assess the promoter activity in soybean after *P. pachyrhizi* infection. Depending on construct availability, four promoters (*pGmRIM*, *pGmASP*, *pgst1* and *pGmCHIT1*) were analysed in our study.

*GmRIM* was shown to be induced by the soybean rust at 72 hpi (see Chapter 1), and *GmASP* was identified as induced by the pathogen at 24 hpi (see Chapter 1). We were not able to highlight a clear/strong induction of these two promoters by Asian soybean rust. We can hypothesize that the level of the GFP proteins three days post-infection with *P. pachyrhizi* was not sufficient to detect any augmentation of the GFP signal. Possibly, it may be necessary to wait longer for an accumulation of GFP proteins allowing an increase of the signal. We can also suppose that the dissection scope used was not powerful enough for the detection of a weak increase of the fluorescence signal. The use of a confocal microscope could be more powerful to reveal a weak augmentation of the GFP fluorescence. In addition, RT-qPCR could be performed to follow more precisely the variation in GFP transcripts under pathogen infection.

Two other promoters, *pgst1* and *pGmCHIT1*, have been investigated in our study and a synthesis of the results is presented in Table 17.

Table 17: Activity profiles of the candidate promoters induced by *P. pachyrhizi*.

Promoter	<i>P. pachyrhizi</i> inoculation	Hormonal treatment			Wounding	Non infected tissues		
		ET	JA	SA		Leaves	Flowers	Roots
<i>pgst1</i>	+ (72 hpi)	+	-	?	-	developed leaves: +	nd	nd
<i>pGmCHIT1</i>	+ (24 hpi)	-	-	?	+	young leaves: - developed leaves: + (vein)	-	+

-: no GFP fluorescence increase; +: GFP fluorescence increase; nd: not done; ?: no conclusion

We observed that the *gstI* promoter from *S. tuberosum* was induced in soybean 72 hours after *P. pachyrhizi* contamination. In *pgstI*:GFP T1 events, an augmentation of the GFP signal was observed around the infection points. The accumulation of GFP proteins and/or transcripts could be followed either by Western blot or RT-qPCR to confirm these results. Investigation of genes involved in plant defense revealed that several G, GCC, W, and GT1 boxes, were present in promoters induced by pathogens or wounding (Rushton, 2002). Interestingly, a cluster of cis-acting elements (W, G and GT1 boxes) was identified by Zou *et al.* in the *gstI* promoter. There is no information on which cis-regulatory elements are sufficient for a pathogen induction, and it would be interesting to investigate this question in soybean under *P. pachyrhizi* contamination.

The *gstI* promoter was induced by ACC, showing that the *gstI* promoter is activated by other stimuli than the rust pathogen. The ethylene pathway is implicated in several other processes than plant defence, including plant growth, flower development and fruit ripening (Iqbal *et al.*, 2017). Therefore, it could be interesting to determine the *gstI* promoter activity in soybean flowers, and during plant development.

The basal expression of this promoter can vary from one species to another. In potato, this promoter had no background activity in non-infected tissues except root apex, but in citrus, the promoter produced a high background level of expression. Therefore, it is not surprising to observe a strong basal expression of the *gstI* promoter in non-infected soybean leaves.

Despite the late induction of *gstI* promoter by the Asian soybean rust (72 hpi) and its induction by ACC, this promoter was selected to drive the barnase expression in order to evaluate barnase/barstar technology.

*GmCHIT1* promoter, controlling the expression of a chitinase, was shown to be activated by *P. pachyrhizi* at 24 h post-inoculation in the first infected soybean leaf cells. This promoter is also locally induced by wounding but not by treatment with an ethylene precursor (ACC) and a methyl jasmonate analogue (coronatime). Other chitinase promoters have been shown to be induced by pathogens and abiotic stresses. The *BjChp* chitinase promoter of *Brassica juncea* has been reported to be induced by *Alternaria brassica*, JA and wounding in *A. thaliana* (Rawat *et al.*, 2017). The promoter of the chitinase AtEP3 of *A. thaliana* was shown to be early induced by *Xanthomonas campestris* but downregulated by wounding (Gerhardt *et al.*, 1997; Takenaka *et al.*, 2009). Different cis-regulatory elements have been identified in the *GmCHIT1* promoter including W and GT1 boxes. It would be interesting to investigate whether the cis-regulating elements found in the *GmCHIT1* promoter are essential and sufficient to trigger a response to *P. pachyrhizi*. To this purpose, a construct with the *GmCHIT1* promoter deleted (1 500 bp

upstream the coding sequence) fused to the GFP, was generated. Unfortunately, the first set of transformation of this construct was not successful due to technical problem in green-house. Finally, the *GmCHIT1* promoter exhibited a low basal expression restricted to the roots and the veins of developed leaves of soybean. This characteristic has been also described with another chitinase promoter of bean (*Phaseolus vulgaris* PvChi4 promoter). PvChi4 promoter has been reported to be expressed in lateral roots and reproductive organs of non-stressed *A. thaliana* plants (Lima *et al.*, 2002).

The basal expression level of the *GmCHIT1* promoter in different soybean tissues together with the induction under rust attack might reflect the potential roles of this chitinase in physiological processes of growth and development, as much as in pathogen protection.

The characteristics of the *GmCHIT1* promoter suggest that it is a candidate of choice for driving barnase gene expression in genetically engineered soybean with the cell death technology. Therefore, this promoter was selected for the development of the barnase/barstar system.



## CHAPTER 3

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EVALUATION OF THE CELL DEATH  
SYSTEM FOR THE CONTROL OF *P.*  
*PACHYRHIZI*



## A. INTRODUCTION

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This chapter concerns the evaluation of the cell death technology used in soybean to control the Asian soybean rust disease. Cell death will be triggered by the expression of two bacterial genes: barnase and barstar. The barnase gene encodes for a ribonuclease that leads to cell collapse when it is expressed, and the barstar gene encodes for a specific inhibitor of barnase. When the barstar interacts with the barnase, a bimolecular complex is formed and the activity site of the barnase is covered (Hartley, 2001). For a proper triggering of cell death, the barnase/barstar ratio must be lower than 1 when the plant is not challenged by the pathogen (*i.e.*, more barstar than barnase) and greater than 1 in the case of infection (*i.e.*, more barnase than barstar). The choice of the promoters controlling the two bacterial genes is important to assess the success of this cell death technology. For this, we placed the barnase encoding gene under the control of a pathogen inducible promoter and constitutive promoters were chosen to control the barstar encoding gene.

The *GmCHIT1* promoter is rapidly induced by *P. pachyrhizi*, possesses low activity in soybean tissues and is not induced following ethylene precursor (ACC) and methyl jasmonate analogue (coronatine) treatments. This promoter appears to be a good candidate to drive barnase expression. The *gst1* promoter (*pgst1*) has been successfully used in the barnase/barstar system in *Solanum tuberosum* against *P. infestans* (Strittmatter *et al.*, 1995). This promoter has been observed induced by *P. pachyrhizi* in soybean. Therefore, it was used as a second candidate to design barnase/barstar constructs.

Different genetic constructions with the selected pathogen inducible promoters (*pGmCHIT1* and *pgst1*) and constitutive promoters (*p35S* and *pNOS*) for the control of respectively the barnase and the barstar were generated. The toxicity of these genetic constructions in absence of contamination by *P. pachyrhizi* was evaluated by transient transformation in *N. benthamiana*. Stable soybean transformations were realised with different genetic constructs to assess if transformed plants could be obtained. Next, the soybean plants generated were evaluated for their resistance to the pathogen.

## B. STUDY OF THE BARNASE/BARSTAR RATIO IN ABSENCE OF INFECTION

### 1) Cloning strategy

The genetic construction we want to introduce in soybean must include barnase placed under the control of a pathogen-inducible promoter, barstar controlled by a constitutive promoter and the HPPD-4 selectable marker gene under the control of the promoter CsVMVM (Figure 55). In order to minimize the impact of the insertion site on transgene expression and to avoid any possible interference between the promoters, the different elements were positioned and oriented as presented in Figure 55.

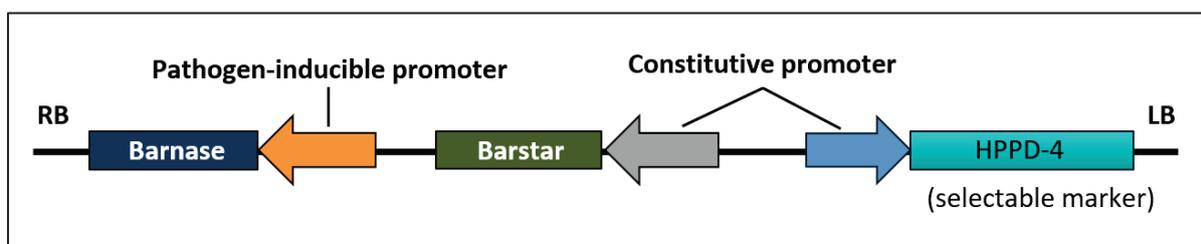


Figure 55: The barnase/barstar cassette. LB: left border, RB: right border

The *GmCHIT1* and *gst1* promoters were selected to drive the barnase expression. However, we were not able to precise whether the level of induction of the *GmCHIT1* or the *gst1* promoter by the pathogen is sufficient to exceed the level of activity of a constitutive promoter controlling the barstar. Therefore, we tested two promoters, the p35S (sequence in Annex 9) and pNOS (sequence in Annex 10) with respectively a strong and a medium activity to drive barstar expression. The following plasmids (Table 18) with the barnase gene under the control of the p*GmCHIT1* or p*gst1* and the barstar gene under the control of either the pNOS or the p35S were cloned.

We first introduced barstar and its promoter (either the p35S or the pNOS) in a construct bearing the HPPD-4 marker (Figure 56-a). The barstar coding sequence under the control of a prokaryotic promoter was introduced outside the RB/LB region to block any possible toxic effects on bacteria growth due to the introduction of the barnase-encoding sequence on the construct (Figure 56-b). Following this step, barnase and the associated pathogen inducible promoter were successfully added to the plasmid obtained (Figure 56-c).

Four cell death plasmids named pBay01404, pBay01405, pBay01834 and pBay01842 were constructed (Table 18). The map of the pBay01834 plasmid is presented in Annex 11 as an example.

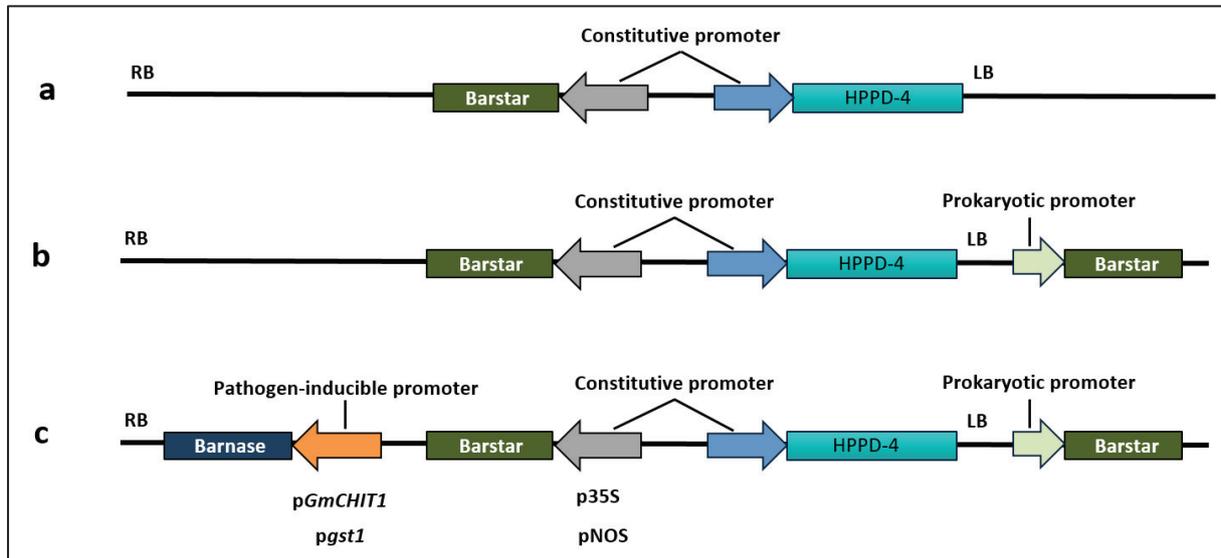


Figure 56: Cloning steps for obtaining the barnase/barstar constructs.

Table 18: Cell death plasmids.

Plasmid name	Barnase promoter	Barstar promoter
pBay01404	<i>pgst1</i>	p35S
pBay01405	<i>pgst1</i>	pNOS
pBay01834	<i>pGmCHIT1</i>	p35S
pBay01842	<i>pGmCHIT1</i>	pNOS

## 2) Evaluation of the construct toxicity in absence of infection

To evaluate the potential toxicity of the constructs, transient transformation of soybean was considered. As this technology was not available in the laboratory, constructs evaluation was conducted in the laboratory of Pr. Ulrich Schaffrath (RWTH Aachen, Germany). However, transient expression by *Agrobacterium* of a construct containing the sole GUS gene revealed an important necrosis of soybean leaves 2 days post transformation (Annex 12). Suggesting that transient expression by *Agrobacterium* in soybean induced a stress of the tissues.

Consequently, the heterologous system *N. benthamiana* was used to evaluate the construct toxicity in a transient system. Infiltration with a construct expressing the GFP under the control of the pCvVMV promoter was tested as a positive control of transient transformation. Four days after infiltration, no necrosis and a strong GFP signal were observed in the infiltrated areas (Figure 57c-d), confirming that the protocol was suitable for the evaluation of the constructs. Likewise no toxicity was observed after transient expression of the barstar encoding gene placed under the control of the NOS promoter (pNOS) indicating that the expected barstar protein was not toxic for the *N. benthamiana* cells (Figure 57-e).

For the constructs with the *gstI* promoter driving the *barnase* expression, chlorosis of the tissues was observed with the pBay01405 construct, whereas the pBay01404 plasmid showed no cell death (Figure 57 h-i). As transient expression of the pBay01404 construct did not trigger any phytotoxicity, we concluded that this construct seems to provide a proper balance of barnase/barstar proteins.

After transient expression of the two constructs pBay01834 and pBay01842 with the barnase under the control of p*GmCHIT1*, a strong chlorosis/yellowing of the infiltrated areas was observed 4 days after infiltration (Figure 57f-g). Therefore, these two constructs appeared phytotoxic for the tissues, and no difference was observable between the use of the 35S and the NOS promoter. The toxicity revealed an imbalance in the barnase/barstar ratio with likely more barnase expressed. As p*GmCHIT1* appears to be the most suitable promoter to control the barnase expression in the barnase/barstar system (see Chapter 2), different experiments were carried out to determine the origin of this phytotoxicity.

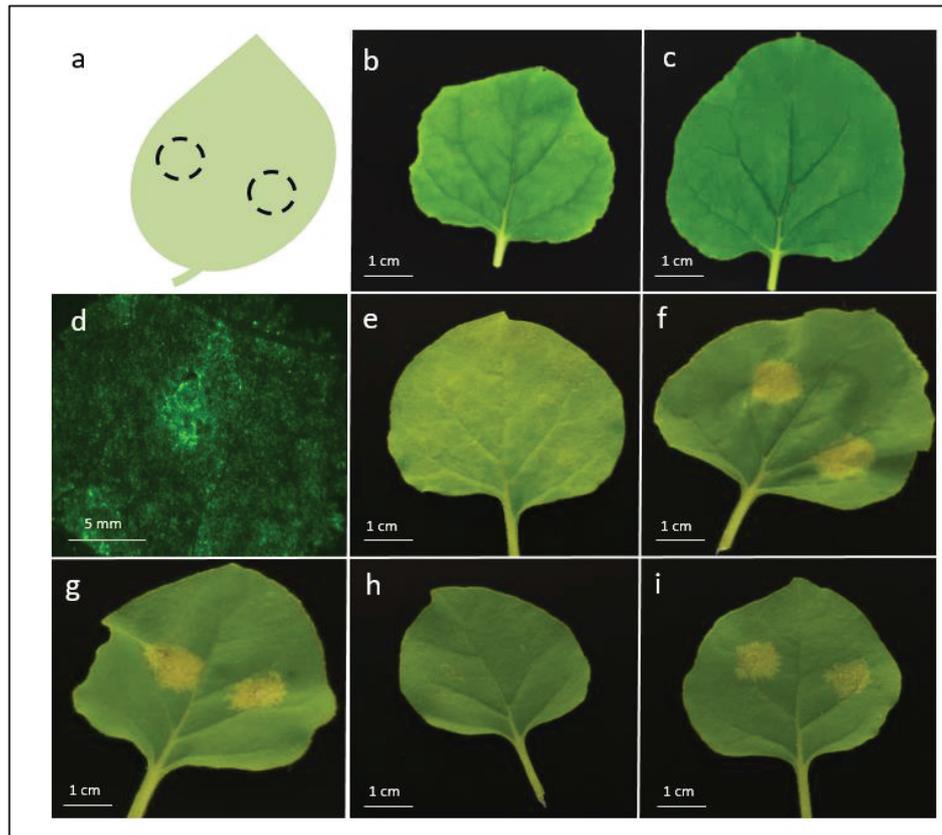


Figure 57: Transient expression of pBay01834, pBay01842, pBay01404 and pBay01405 constructs in *N. benthamiana*. Pictures were taken 4 days after agro-infiltration. a) Schematic representation of the infiltrated area (circles) on *N. benthamiana* leaves. The results after infiltration with b) the buffer solution, c) and d) the pCsVMV:GFP, e) the pNOS:barstar, f) the pBay01834, g) the pBay01842, h) the pBay01404 and i) the pBay01405 constructs. d) GFP spot observed with a dissection scope (Leica Z16 APO) under GFP filter.

### **pGmCHIT1 and p35S expression after transient transformation**

Considering the observed phytotoxicity, we could suppose a high expression of the barnase in absence of *P. pachyrhizi* infection resulting from a high expression of the *GmCHIT1* promoter. To test this hypothesis, the pGmCHIT1:GFP and the p35S:GFP constructs were transiently expressed in *N. benthamiana* and the GFP fluorescence was quantified 4 days post-infiltration (Figure 58). The results showed that a light fluorescence was detected following pGmCHIT1-GFP transient expression, revealing a weak activity of the *GmCHIT1* promoter in *N. benthamiana*. As the fluorescence intensity measured with p35S:GFP was higher than that observed with pGmCHIT1:GFP, we concluded that pGmCHIT1 activity after transient transformation in *N. benthamiana* is lower than p35S activity. We hypothesize that in barnase/barstar constructs pGmCHIT1 activity is influenced by other promoters.

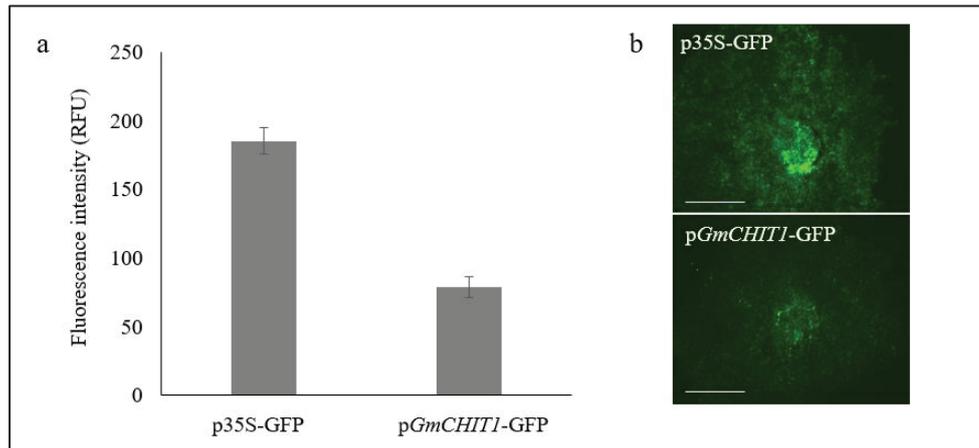


Figure 58: **GFP expression under the control of *GmCHIT1* or 35S promoters after transient expression in *N. benthamiana*.** Observations were performed 4 days post agro-infiltration. a) Fluorescence intensity in relative fluorescence units (RFU) measured after transient expression of p35S:GFP and pGmCHIT1:GFP constructs. Mean of 12 replicates  $\pm$  SD is shown. b) Representative images were observed with a dissection scope (Leica Z16 APO) under GFP filter. Scale bar: 5 mm.

### **Improvement of the cell death constructs**

It is possible that the constitutive promoters driven by either the barnase or the HPPD-4 genes could still influence the *GmCHIT1* promoter activity. Insulator elements are useful tools to avoid interferences between promoters (Singer *et al.*, 2012). Therefore, an insulator sequence (500 bp from the phage  $\lambda$ ) that have shown to be functional in other constructs (Bayer personal data), was added between the *GmCHIT1* promoter and the barnase gene coding sequence. In addition to increase barnase translation, a short sequence of 129 bp from the 5' non-translated region of the *N. benthamiana* etch virus (5' TEV) was added after the 35S promoter of the barnase. Indeed, Carrington and Freed (1990) showed that this 5' TEV sequence dramatically stimulated translation. The new construct obtained was named pBay02475 (Figure 59-a) and was then transiently expressed in *N. benthamiana* to assess its associated phenotype. The same controls as those presented in Figure 57 were used (*i.e.*, B, C, D and E observations). The same phenotype of tissue chlorosis was observed following transient expression of the pBay01834 and pBay02475 constructs (Figure 59-b), indicating that even with the addition of an insulator and the 5' TEV sequence, the barnase/barnase construct was still phytotoxic.

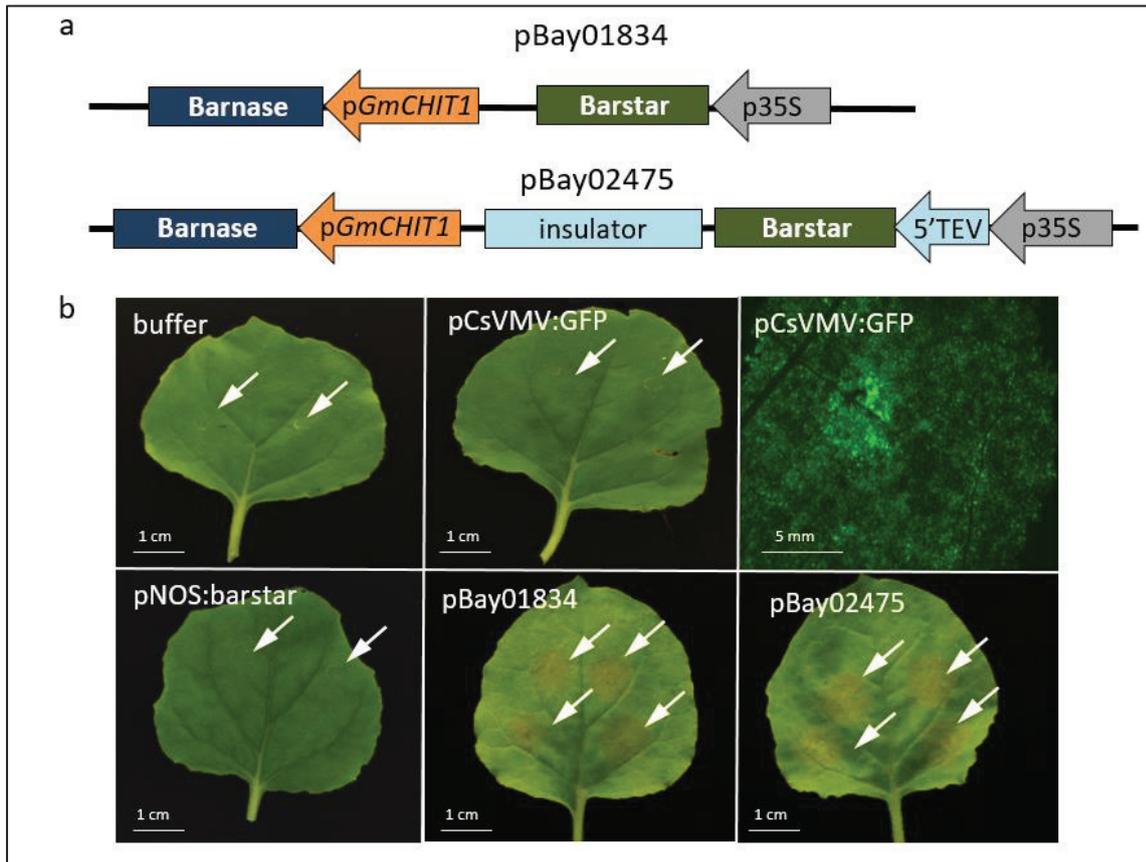


Figure 59: **Transient expression of pBay01834 and pBay02475 constructs in *N. benthamiana*.** a) Schematic representation of the pBay01834 and pBay02475 constructs. In blue, the elements added to improve the construct. b) Observation of the phenotype 4 days post-infiltration. The plasmid pBay01834 was used as a reference. Scale bar: 1 cm. Arrows show the infiltrated areas.

It was likely that the toxicity observed with the pBay01834, pBay01842 and pBay02475 was due to a higher quantity of barnase than barstar. However, the reasons for the probable imbalance in the barnase/barstar ratio are still unknown.

*N. benthamiana* is an easy and fast heterologous system and results can be considered as an indication of what we may expect in soybean. Nevertheless, generation of stable transgenic soybeans was evaluated to assess if the toxic phenotype observed in *N. benthamiana* could be also observed in soybean.

## C. SOYBEAN TRANSFORMATION

To check the possibility of generating soybean plants, a stable *Agrobacterium*-mediated transformation was performed with the two constructs containing the *GmCHIT1* promoter that showed a toxic phenotype or the pBay01404 plasmids with the *gstI* promoter, which is non-phytotoxic in *N. benthamiana*.

### 1) The constructs with the *GmCHIT1* promoter

After transformation with the pBay01834 and pBay01842 plasmids, positive shoots (resistant to the TBT herbicide) were recovered on the selectable medium (Figure 60-a). However, the obtained shoots were weakly and less developed than positive shoots obtained with a plasmid expressing the GFP (Figure 60-a). After one month on elongation medium, the small positive shoots generated with the barnase/barstar constructs were transferred to rooting medium and later to a greenhouse. Only one shoot by construct survived more than one week in the greenhouse. The T0 seedlings from those unique events expressed the HPPD (Figure 60-b), but they did not develop properly and died after two weeks in the greenhouse.

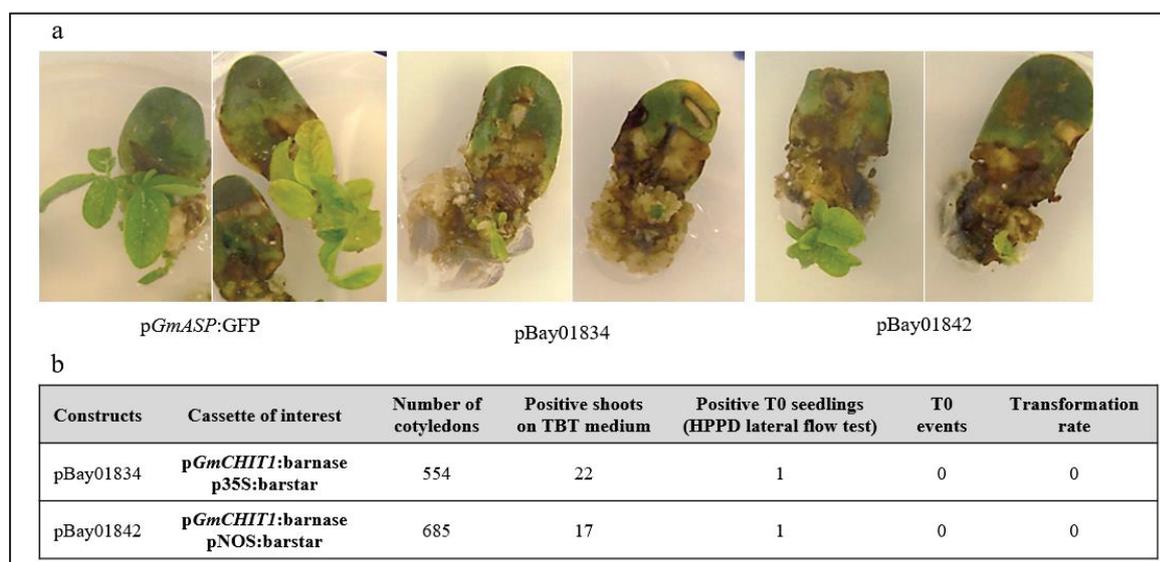


Figure 60: Soybean transformation with pBay01834 and pBay01842 containing the *GmCHIT1* promoter. a) Positive shoots (on TBT medium) obtained with the p*GmASP*:GFP, pBay01834 and pBay01842 constructs. b) Results of the selection of positive transformed plants.

No transformed soybean events were obtained after transformation with the two barnase/barstar constructs. No difference was observed between the use of the p35S and the pNOS for barstar expression. These results revealed that the phytotoxicity of the pBay01834 and pBay01842 constructs observed in *N. benthamiana* was probably also present in soybean as no viable events were recovered.

## 2) The construct with the *gstI* promoter.

Following soybean transformation with the pBay01404 construct, positive shoots (resistant to the TBT herbicide) were obtained. The shoots did not show any reduction of growth and were similar to shoots obtained with a construct expressing the GFP (*pGmASP:GFP*) (Figure 61-a). The shoots developed normally on the selection medium. After the HPPD lateral flow test, 49 positive T0 seedlings were obtained, giving 49 T0 events. A transformation efficiency of 4% was reached, which correlated with the transformation efficacy obtained with plasmids containing the GFP (see Chapter 2, page) (Figure 61-b).

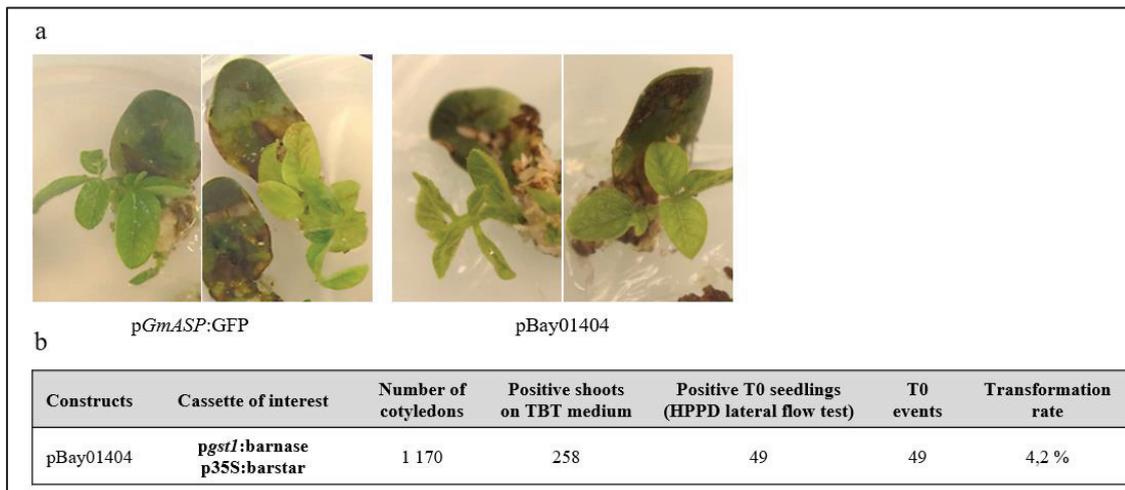


Figure 61: Soybean transformation with pBay01404 constructs containing the *gstI* promoter. a) Positive shoots (on TBT medium) obtained with the *pGmASP:GFP* and pBay01404 constructs. b) Results of the selection of positive transformed plants.

## D. BARNASE/BARSTAR SYSTEM EVALUATION IN SOYBEAN

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Stable T0 events with the plasmid pBay01404 (*gstI* promoter controlling the barnase and p35S promoter controlling the barstar) were generated.

### 1) Qualitative and quantitative evaluation of the T0 events resistance to *P. pachyrhizi*.

Among the 49 T0 events positive for the HPPD gene, 25 events were analysed to check the presence of barnase/barstar genes in the plants. For this, a PCR approach was developed to detect barnase, barstar and the HPPD genes. To be sure that the amplification observed was not the result of the *Agrobacterium* contamination, amplification of a sequence outside the LB/RB region of the pBay01404 plasmid was carried out. Fourteen events showed amplification of the barnase-, barstar- and HPPD-encoding sequences and no amplification of the sequence outside the LR/RB region of pBay01404 (see Annex 13). Eight events showing a strong HPPD lateral flow test response were selected for the evaluation of their resistance to *P. pachyrhizi*. WT plants were used as negative control, and a line containing the *Rpp5* gene (Inox line provided by Bayer Brazil) was used as positive control. Under rust contamination, the Inox plants produced a hypersensitive response and developed RB-type lesions. Four detached leaves of the selected plants were inoculated with the pathogen spores, and the symptoms were observed at 8, 12 and 14 dpi. First observations revealed that at all the times, the contaminated leaf surface seemed to be more important in the T0 events compared to the WT. Similar results were observed for the 4 detached leaves of each individual plant and for the all the tested T0 events. The Inox plants exhibited RB lesions as expected (Figure 62).

To precisely describe the phenotype, the number of uredinia formed was counted for 10 inoculation points per leaf. At 8 dpi, 3 T0 events (19, 20 and 24) showed a reduced number of uredinia compared to that in the WT plants, with a similar number to those observed with the Inox line (Figure 63). This finding reflected a potential impact of the construct on the pathogen development. These 3 events were kept for further analysis.

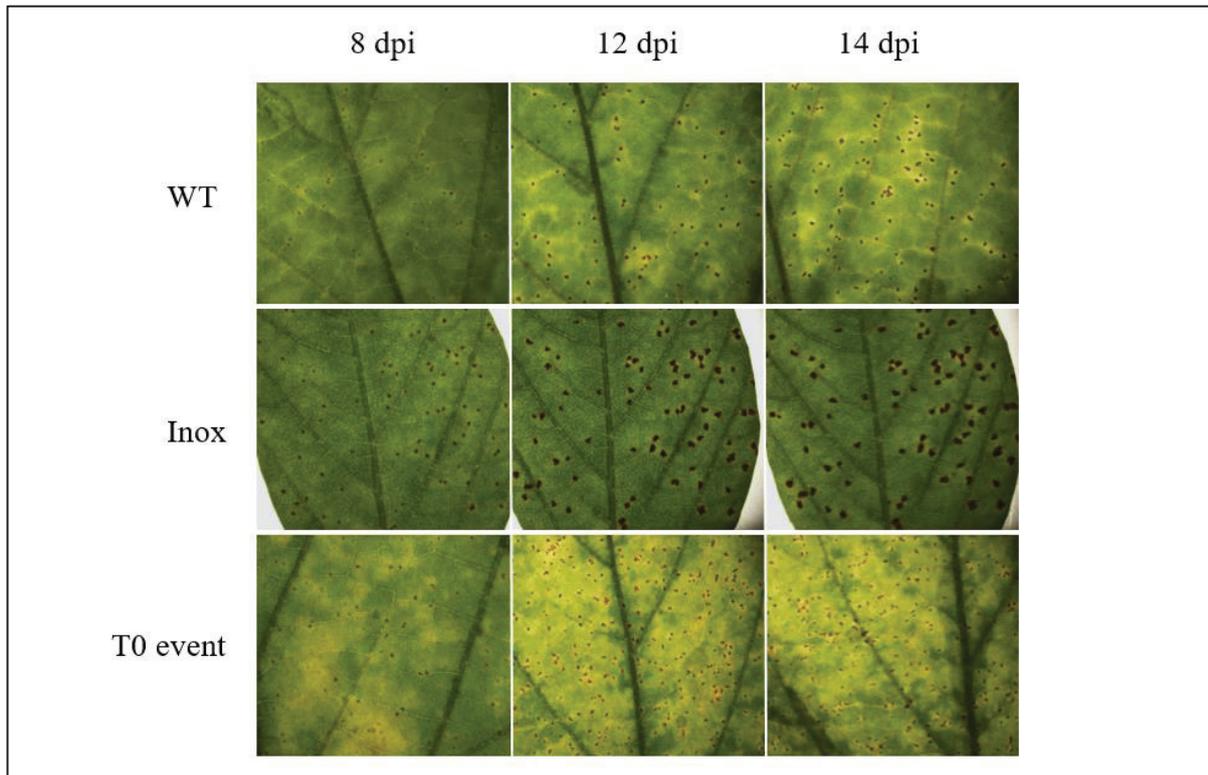


Figure 62: **Macroscopic observations following *P. pachyrhizi* infection on pBay01404 T0 events.** Representative pictures of results obtained with WT, Inox and T0 plants. Detached leaves were observed at 8, 12 and 14 dpi.

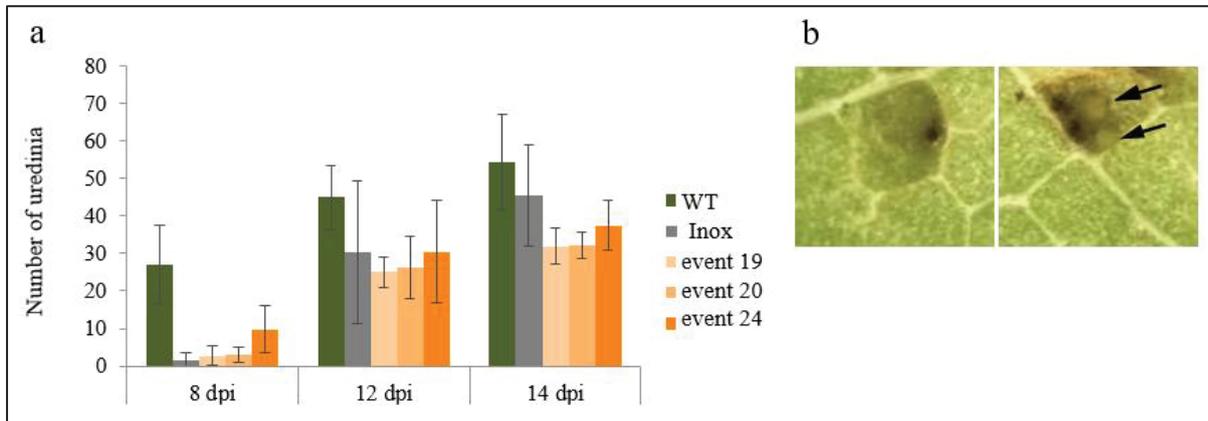


Figure 63: **Number of uredinia following *P. pachyrhizi* inoculation.** a) Mean of the uredinia number on 10 inoculation points per leaves. 4 biological replicates +/- SD. Uridinia were counted at 8, 12 and 14 days post-contamination, on WT, Inox and 3 pBay01404 T0 events (19, 20 and 24). b) Left: inoculation point without uredinia. Right: inoculation point with two uredinia (arrows).

## 2) Evaluation of the T1 events resistance to *P. pachyrhizi*

To confirm these preliminary observations on the 3 T0 events, *P. pachyrhizi* contamination was repeated on the following T1 generation on entire plants. For this experiment, between 12 and 16 sister plants by event were contaminated with the pathogen. The number of transgene copy on plants of each independent T1 events was assessed by ddPCR revealing that the inoculated plants were heterozygous for the transgene. The surface of infection (% of leaf surface covered with symptoms) was assessed to determine whether a reduction of the diseased leaf area could be observed. WT plants were used as negative control. On the 3 T1 events and the WT plants, the infection observed at 13 dpi varied between 30 and 50%, revealing no difference between the soybeans transformed with the pBay01404 construct and the WT (Figure 64 a-b). Macroscopic observations showed that the pathogen was able to sporulate on the WT and the 3 T1 events (Figure 64 c). Inox plants were used as a control of infection, and RB lesions were observed as expected (data not shown).

T0 soybean events were generated with the pBay01404 plasmid. A first evaluation of the T0 events' resistance to *P. pachyrhizi* was performed, and reduced growth of the fungus was observed on 3 T0 plants. The corresponding T1 plants were inoculated by the pathogen. The first results showed that no reduction of the infection was observed on those T1 plants compared to WT soybean, and the pathogen was able to sporulate.



## E. DISCUSSION

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Following the evaluation of 4 candidate promoters, the *gst1* and *GmCHIT1* promoters were identified as induced by Asian soybean rust. According to our criteria, the *GmCHIT1* promoter was shown to be the more appropriate to control barnase expression. This promoter locally activated by *P. pachyrhizi* possesses a low activity in soybean tissues and is not induced by ET and JA hormonal treatments.

Two “cell death plasmids” were generated with barnase placed under the control of the *GmCHIT1* promoter and barstar under the control of the p35S or the pNOS (pBay01834 and pBay01842 plasmids, respectively). The toxicity of the constructs was evaluated in the absence of the pathogen by transient transformation in *N. benthamiana*. In both cases, phytotoxicity was observed, suggesting an overproduction of barnase not counterbalanced by barstar. Following transient expression of the genetic constructions, evaluation of this ratio based on the detection of barnase and barstar mRNA may be not technically possible due to the ribonuclease activity of the barnase (Strittmatter *et al.*, 1995). Therefore, detection of the barnase and barstar proteins was investigated using immuno-detection (see Annexe 14). However, barnase proteins were not detected following transient expression in *N. benthamiana* of pBay01834 and pBay01842. A Western blot using specific antibodies against the barstar did not give clear results on the protein detection. Additional work is needed to detect the barnase and barstar proteins by Western blot and appreciate the ratio of these proteins in the transformed plants. Finally, RT-qPCR could be realized following transient expression of the pNOS-barstar plasmid, to assess if the gene is well transcribed in *N. benthamiana*.

On the pBay01834 construct, an insulator sequence was added between the p*GmCHIT1* promoter and the barstar to suppress any potential interaction of the transgene with the inducible promoter (Hasegawa and Nakatsuji, 2002; Singer and Cox, 2013). Moreover, the 5'TEV sequence was added after p35S to boost the barstar translation. However, phytotoxicity was still observed likely because the barnase/barstar ratio was greater than one.

It is possible that even with the modifications, the constitutive promoters from barstar or HPPD still influence the p*GmCHIT1*. To evaluate the effect of the insulator, we could compare the fluorescence signal obtained after transient transformation of the pBay01834 and pBay02475 constructs, both with the barnase substituted by the GFP. Likewise, we could assess the GFP fluorescence of the p35S:GFP and the p35S5'TEV:GFP constructs after transient

transformation in *N. benthamiana* in order to appreciate an increase of translation due to the 5'TEV sequence

Finally, different constitutive promoters such as the pCsVMV, could be used to control the barstar expression. The CsVMV promoter has shown to be more active than the p35S following transient transformation in *N. benthamiana* (see Annexe 15). Increasing the quantity of barstar proteins could solve the problem of toxicity due to the constructs. Nevertheless, we must be aware that in the case of a very high barstar expression, the induction of the *GmCHIT1* promoter may not be sufficient to exceed the barstar quantity and allow ribonuclease activity.

We were not able to obtain stable transformed soybean with the toxic constructs, revealing a correlation between the results obtained with *N. benthamiana*. It is likely that an excessive amount of barnase protein may be toxic for the soybean shoots during and after transformation. This toxicity could explain why the shoots were unable to grow properly and died rapidly after their transfer to the greenhouse.

In parallel to the use of the *GmCHIT1* promoter in the cell death system, the *gstI* promoter from *S. tuberosum* was used to control the barnase expression. Two constructs with the barnase under the control of the *gstI* promoter and the barstar under the control of the p35S or the pNOS were generated. After transient expression in *N. benthamiana*, we observed no phytotoxicity when the barstar was under control of the 35S promoter (construct pBay01404).

T1 plants derived from the 3 T0 events showing a reduction in the pathogen growth were assessed for their resistance to *P. pachyrhizi*. No difference in the surface of infection was observed at 13 dpi compared to WT plants, and the pathogen was able to sporulate on the plants. To precise the fungal development in the T1 plants, it would be interesting to observe more in detail the pathogen development and to assess the number of uredinia formed at 8 dpi. Langenbach *et al.*, 2013, quantified *P. pachyrhizi* mRNA in transgenic soybeans expressing *Arabidopsis* genes linked to the NHR against Asian soybean rust in order to assess the resistance of those plants to the pathogen. They normalized the abundance of *P. pachyrhizi* mRNA transcript of the  $\alpha$ -tubulin gene (PpTUB) to the soybean mRNA transcript of *GmUBQ3*. The same method could be applied to the plants of the 3 T1 lines for a precise assessment of the fungal development on the plants. Furthermore, more T1 lines could be evaluated for *P. pachyrhizi* resistance to potentially identify other plants exhibiting a reduced pathogen development.

In parallel of the test of resistance to Asian soybean rust, the T1 plants obtained could also serve for the global evaluation of the cell death system. We show that the *gstI* promoter was strongly induced by an ACC treatment in soybean (see Chapter 2). Consequently, an ACC treatment

could be realized on the T1 plants to test whether *pgstI* induction is sufficient to trigger plant cell death. The potential cell death could be stained with trypan blue dye (Strober, 2001) and Western blots using antibodies against the barnase and barstar proteins could be performed in order to assess the barnase/barstar ratio with and without ACC treatment. Different biochemical processes are associated with the HR. ROS, as the hydrogen peroxide ( $H_2O_2$ ), are massively produced, intracellular calcium level is modified, antimicrobial compounds are released, and SA accumulation is observed (Iakimova *et al.*, 2005). The cell death potentially obtained after an ACC treatment could be analysed in order to define whether certain characteristics in common with an HR are also triggered. For instance,  $H_2O_2$  could be stained with a 3,3'-diaminobenzidine (DAB) solution (Daudi and O'Brien, 2012).

Obtaining soybean plants with the pBay01404 construct opens the way for a better comprehension of the barnase/barstar system. However, more investigations are necessary to evaluate the potential of this approach for *P. pachyrhizi* control.



# GENERAL DISCUSSION

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## GENERAL DISCUSSION

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Asian soybean rust stands as the most destructive foliar disease of soybean (Echeveste da Rosa, 2015). Brazil, one of the leading soybean producing countries, is impacted by the disease each year (Godoy *et al.*, 2016). The control of *P. pachyrhizi* is essentially based on fungicidal treatments (Langenbach *et al.*, 2016a; Miles *et al.*, 2007) and breeding solutions are unlikely (personal communication).

In this context there is an urgent need of new solutions, and biotechnological approaches are currently evaluated by the agrochemical/breeding industry. Transgenic approaches for disease control can target either the plant defense mechanisms or the fungus. In both cases, the choice of the right promoter to control the selected gene is an essential point. Pathogen inducible promoters allowing transgene expression only when and where it is needed, are essential tools for the development of such strategies. The project of my thesis was oriented on the identification and study of promoters inducible by *P. pachyrhizi* and their use in a cell death system, a new biotechnological approach for the Asian soybean rust control.

### 1) From pathogen inducible promoters to synthetic promoters

Five candidate promoters potentially induced by *P. pachyrhizi* were identified using transcriptomic and bibliographic data. Stable transgenic soybeans expressing the different promoter:GFP transcriptional fusions were generated to evaluate the activity of the candidate promoters in presence of the Asian soybean rust. The *GmCHIT1* promoter (p*GmCHIT1*) from *G. max* encoding a chitinase and the *gst1* promoter (p*gst1*) from *S. tuberosum* encoding a glutathione S-transferase were induced by *P. pachyrhizi*. Most of the pathogen inducible promoters are often induced by other stimuli (Gurr and Rushton, 2005b). The activity of these two promoters following hormonal treatments and mechanical wounding were studied. A treatment by an ethylene precursor induced the p*gst1* and the p*GmCHIT1* promoter showed to be locally activated by wounding. One possible strategy to overcome these inductions by several abiotic stimuli would be to produce synthetic promoters 100% responsive to the sole presence of the pathogen.

Synthetic and inducible promoters are designed by using the 35S promoter core region (essential to initiate transcription) in combination with different cis-regulatory elements (Cazonelli and Velten, 2008). In *A. thaliana*, synthetic promoters containing tetramers of a single type of element (boxes W1, W2, GCC, JERE, S, Gst1, and D) have shown to mediate local gene expression after bacterial and fungal pathogen (*P. syringae* and *Erysiphe cichoracearum*) attacks (Rushton *et al.*, 2002). Moradyar *et al.*, (2016) constructed a synthetic promoter (SP-DDE) highly responsive to fungal pathogen and carrying dimerized forms of D and E cis-acting elements upstream of the 35S minimal promoter. The expression of a chitinase under the control of the SP-DDE synthetic promoter conferred resistance to *S. sclerotiorum* in transgenic canola (*Brassica napus* L.).

The *GmCHIT1* and *gst1* promoters have shown to possess common cis-regulatory elements. Three W and GT1 boxes were identified by Zou *et al.*, (2014) in the *gst1* promoter and we identified 21 W boxes and 10 GT1 boxes in the *GmCHIT1* promoter. In the soybean promoter of the calmodulin *SCaM-4*, a GT1 cis-element was identified as essential for induction by *P. syringae* pv *tomato* and NaCl in *A. thaliana* (Park, 2004). In the *ChiIV3* chitinase promoter of pepper, one W-box was described as essential to trigger the induction after *Phytophthora capsici* contamination (Liu *et al.*, 2017). W-box refers to the binding site of WRKY transcription factors (Eulgem *et al.*, 2000) and in soybean these regulators have been shown to be implicated in the response to *P. pachyrhizi* (Bencke *et al.*, 2014).

Deletion of the *GmCHIT1* promoter (3 454 bp) and the *gst1* promoter (273 bp) could be realized in order to identify the minimal sequence and the associated cis-elements necessary for the induction by the pathogen. Once the motifs of interest selected, motif copy number and spacing have to be improved for a better optimization of temporal and spatial expression pattern (Liu and Stewart, 2016; Venter, 2007). Different combinations of cis-regulatory elements, identified as necessary for the Asian soybean rust induction, could be assembled and tested for the optimal design of a synthetic promoter strongly and strictly induced by *P. pachyrhizi*.

Liu *et al.*, (2014) discovered soybean cyst nematode (SCN)-inducible motifs among promoters of 18 co-expressed soybean genes selected from six microarray studies performed during a compatible soybean–SCN interaction. Among them, promoters of two genes were identified as induced by SCN at 3 days post infection. The authors identified 16 overlapping motif regions (OMRs) using bioinformatic tools in those two SCN-inducible promoters. Among the 16 OMRs, 11 OMRs were experimentally confirmed to be SCN-inducible, leading to the discovery of 23 core motifs of 5- to 7-bp length (Liu *et al.*, 2014).

A same strategy could be applied for detection of *P. pachyrhizi* inducible motifs. In this work, 678 genes upregulated by the pathogen at 24 hpi were identified through the analysis of transcriptomic data. Other studies also identified soybean genes induced at this time of infection in the case of a compatible interaction (van de Mortel *et al.*, 2007; Schneider *et al.*, 2011) and could serve for identification of common induced soybeans genes. Among them, genes with similar transcript profiles could be selected as co-regulated genes. Promoters of these co-expressed genes could be studied and OMRs identified for discovery of a core motif induced by *P. pachyrhizi* that will serve for synthetic promoter design.

Study of soybean cis-regulatory elements are often realized in heterologous system, using transient transformation or hairy roots ensuring rapid results (Chai *et al.*, 2013, 2016; Hernandez-Garcia and Finer, 2016). However, *P. pachyrhizi* infection of *A. thaliana* or *N. benthamiana* does not result in a compatible interaction, the pathogen does not infect soybean roots and transient transformation of soybean leaves is not efficient. Therefore, evaluation of promoter's activity following *P. pachyrhizi* infection is limited to the use of stable transgenic soybean, requiring significant time before yielding results.

A synthetic promoter highly and rapidly induced by the Asian soybean rust would be appropriate for biotechnological approaches.

## **2) Induction of cell death to mimic an HR: is the barnase/barstar system efficient for *P. pachyrhizi* control?**

One method to develop transgenic plants with increasing disease resistance is to produce one of the component of the HR, cell death, requiring precise transgene expression. Artificial cell death was previously developed in potato to control *Phytophthora infestans*. The technology was based on a barnase ribonuclease highly toxic for the plant cell. This toxicity required a strict control of the barnase gene expression that had to be induced only in the presence of the pathogen. The lethal expression of the barnase was counterbalanced by barstar a highly specific inhibitor of the barnase. This technology was evaluated in this work to control *P. pachyrhizi*. A critical point was the modulation of the ratio of barnase/barstar that must change during the infection. For this purpose, the barnase was placed under the control of an inducible promoter and the barstar under the control of a constitutive promoter.

Molecular constructs with different combinations of constitutive (p35S and pNOS) or inducible (p*GmCHIT1* and p*gstI*) promoters, selected to drive respectively the barstar and barnase gene expression, were generated. *Nicotiana benthamiana* was used to evaluate the construct toxicity in absence of the pathogen. One single construct containing the fusion p*gstI*:barnase was shown to be non-phytotoxic. The toxicity observed with the majority of evaluated combinations was probably due to an over-expression of the barnase ribonuclease and/or a low quantity of barstar. Western blots of barnase and barstar proteins were realized but the first experiments did not give clear results on the protein detection. An RT-qPCR on the barstar mRNA following the expression of the pNOS: barstar construct could be conducted to precise if the gene was properly transcribed in *N. benthamiana* and in soybean. In the case of a low barstar expression, a study should be conducted to improve its expression.

The non-toxic construct was successfully introduced in soybean plants whereas the constructs showing toxicity in *N. benthamiana* didn't allow the obtention of transgenic soybeans. Generated soybeans were challenged with rust, but no significant resistance was observed on three independent T1 lines. To get deeper data, the quantification of the fungus could be assessed by RT-qPCR to precise if the fungal development has been affected in these events. Quantification of the cell death components should be conducted to understand the lack of protection. Finally, additional T1 lines are available and they could be tested for their level of resistance.

*N. benthamiana* model is efficient to observe the toxicity of the barnase/barstar constructs in absence of the pathogen. The whole set of the 23 genes highly induced by *P. pachyrhizi*, selected by transcriptomic data analyses, could be also tested as additional potential candidates to reach a "Proof of Concept". Assuming that the expression of the barstar gene is the limiting step, other constitutive promoters have to be used. For exemple, the CsVMV promoter (Samac *et al.*, 2004) and the promoters controlling the ubiquitin soybean genes (Hernandez-Garcia *et al.*, 2010) have been described as efficient constitutive promoters. Thus, other "barnase/barstar" molecular constructions with different constitutive and inducible promoters could be tested in *N. benthamiana* to select only those showing no toxicity for stable soybean transformation.

To conclude, further investigations are necessary to better understand the functionality of the barnase/barstar system and evaluate its potential and limits for the control of *P. pachyrhizi*.

In addition to the barnase/barstar system, other strategies for cell death induction might be considered. A system based on the expression of an *R* gene and the corresponding *Avr* could be developed. The advantage of this technology is that a full HR response including cell collapse

and the associated biochemical mechanisms (ROS production, modification of the  $\text{Ca}^{2+}$  level, transcriptional reprogramming etc...) could be triggered. The Avr/R genes controlled by a *P. pachyrhizi* inducible promoter, should be induced only in presence of the pathogen. Different couples of Avr/R genes could be used. The effector protein AvrPto of *Pseudomonas syringae* pv *tomato* triggers ETI and cell death in tomato (*Solanum lycopersicum*) cultivars that are carrying an effector recognition complex composed of a protein kinase (Pto) and a R protein of the NB-LRR family (Prf) (Zipfel and Rathjen, 2008). Transient expression in *N. benthamiana* plants of Pto and AvrPto triggers a HR (Sessa *et al.*, 2000) and a similar scenario could be considered in soybean. In soybean, Rpg1r NB-LRR protein detects the AvrRpm1 protein of *Pseudomonas syringae*. When these two proteins are transiently co-expressed in *Nicotiana glutinosa* (that lack the R gene for AvrRpm1 recognition), a strong cell death is observed (Ashfield *et al.*, 2014). Both of this Avr/R couple could be evaluated for Asian soybean rust control.

A system of one component could also be considered by expressing a single gene that induces cell death. The *A. thaliana* peptide kiss of death (KOD) of 25 amino-acids was identified as an inducer of programmed cell death (Blanvillain *et al.*, 2011). This peptide is implicated in PCD of the suspensor (a single file of cells that support embryo development) and root hairs. The overexpression of this peptide in *N. tabacum* is sufficient to induce PCD (Blanvillain *et al.*, 2011). A first experiment was realized by transiently overexpressing this peptide (construct p35S:KOD::GFP kindly provided by Patrick Gallois) in *N. benthamiana* by no cell death was observed in our conditions.

### 3) Other biotechnological solutions to control the Asian soybean rust?

Different transgenic strategies have been or are currently evaluated for the Asian soybean rust control. The non-exhaustive Table 19 summarizes the advantages and limits of some approaches.

So far, the most promising results were obtained with the expression of the *CcRpp1* gene from *Cajanus cajan* (Kawashima *et al.*, 2016b). A wise strategy would be to develop soybeans with several genes that use different mechanisms to confer resistance to secure the durability of a commercial product. This requires constant research on the evaluation of biotechnological systems for *P. pachyrhizi* control.

Table 19: Advantage and limit of approaches developed for *P. pachyrhizi* control.

Approach	Example	Advantage	Limit
RNAi (HIGS or ectopic spray)	Any essential genes	Stacking of genes to increase efficacy	No proof of concept clearly achieved. Efficacy ?
Expression of antimicrobial compounds	Some examples mentioned in the literature.	Peptide might be optimized. Durability if unspecific mode of action.	Phytotoxicity, efficacy ?
Expression of <i>R</i> gene from unrelated plant species	<i>CcRpp1</i> , a <i>R</i> gene from <i>Cajanus cajan</i> .	Efficacy. Stacking possible	Durability of a single gene
Expression of NHR gene	<i>CL4</i> is implicated in NHR of <i>A. thaliana</i> against <i>P. pachyrhizi</i> .	Provide resistance against <i>P. pachyrhizi</i> . Could lead to durable resistance.	Level of resistance (may required several genes for a strong resistance). Plant fitness
Signal regulation	Soybean WKY transcription factors are implicated in <i>Rpp2</i> resistance.	Activation of a large number of genes.	No GM soybean expressing those genes have been generated.
Modulation of the defense pathway	Expression of <i>CASAR82A</i> (required for SAR induction in <i>Capsicum annuum</i> ).	Provide resistance against <i>P. pachyrhizi</i> .	Might be very challenging to handle (efficacy versus plant fitness).

Other methods for plant disease engineering, not yet applied to *P. pachyrhizi*, have been proposed.

Neutralizing fungal virulent products can increase disease resistance. For example, *Sclerotinia sclerotiorum* secretes oxalic acid during plant tissue invasion. This organic acid has different functions including lowering the pH for an optimal activity of cell-wall degradation enzymes, suppression of the oxidative burst and weakening of the plant cell walls *via* calcium chelation (Cessna *et al.*, 2000). Enzyme(s) that can degrade oxalic acid appeared as another option for plant disease engineering. In soybean, overexpression of an oxalate decarboxylase degrading oxalic acid provided resistance to *Sclerotinia sclerotiorum* (Cunha *et al.*, 2010).

In the same objective, *P. pachyrhizi* virulent products could be neutralized. Effectors of the Asian soybean rust targeting the soybean defense have been identified (Qi *et al.*, 2017), and the availability of the genome will open the way to the discovery of other fungal effectors/virulence factors of the pathogen. Any technology annihilating the “effect” of the effectors (as blocking their entrance in the host plant for instance) could be considered for disease control (Kale *et al.*, 2010).

Transfer of PRRs that detects common microbial products into plant species that lack them, represents also a promising approach to develop a durable resistance (Dangl *et al.*, 2013). For instance, the Arabidopsis PRR ER-receptor (ERF) recognizes the EF-Tu elongation factor of bacteria. Expression of ERF in *Nicotiana benthamiana* and *Solanum lycopersicum*, which

cannot recognize Ef-Tu, conferred resistance to different pathogenic bacteria (*Ralstonia solanacearum* and *Xanthomonas perforans*) (Lacombe *et al.*, 2010). Following the same principle, transfer of the tomato *Ve1* gene encoding a PRR, conferred *Verticilium* resistance to *Arabidopsis* (Fradin *et al.*, 2011). However, no GM plants resulting from this approach have been yet developed by the industry.

Pathogens are able to leverage on host plant's gene expression to increase their fitness and promote disease. These genes can be seen as Susceptibility (*S*) genes. *S* genes can be useful sources of breeding disease resistance and their loss or alteration of function would promote resistance (Dangl *et al.*, 2013). In Barley, the *MLO* gene is required for the powdery mildew invasion and loss-of-function of this gene results in resistance to this pathogen. *mlo*-resistance have been described in many other plant species. For instance, silencing of *MLO* orthologues in pepper (*Capsicum annuum*) or apple (*Malus domestica*) conferred resistance to powdery mildew species adapted to those plants. Yet, pleiotropic effects are often associated with *mlo*-mediated immunity, which can harm plant yield and quality (Kusch and Panstruga, 2017).

The induction of plant cell death was most challenged than expected. This did not allow us to achieve a "Proof of Concept" of the technology. More investigations are required in light of what has been observed. Nevertheless, this work allowed the identification of a new soybean promoter induced by the Asian soybean rust (WO2018217474 patent, Cabre *et al.*, 2018) that could be used for the development of biotechnological approaches for *P. pachyrhizi* control.

In the future, a better understanding of the soybean interaction with *P. pachyrhizi* may provide new knowledge for the development of transgenic strategies for the control of this disease and may open the route to disruptive technologies.



# ANNEXES

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Annex 1: Sequence alignment of *Gm11G170300*, *Glyma11G171400*, *Glyma18G06110*, *Glyma02G228100* and *Glyma14G195000*. Alignment realized with Vector NTI software.

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CDS_Glmyma11G170300 (1) ATGTGTGGCATACTTGCTGTCTTGTTGCTCTGATTCATCTCAAGCCAA
CDS_Glyma.11G171400 (1) ATGTGTGGCATACTTGCTGTCTTGTTGCTCTGATTCATCTCAAGCCAA
CDS_Glyma.18G061100 (1) ATGTGTGGCATACTTGCTGTCTTGTTGCTCTGATTCATCTCAAGCCAA
CDS_Glyma.02G228100 (1) ATGTGTGGTATTCTTGCTGTCTTGTTGCTCTGATGACTCTCAGCCAA
CDS_Glyma.14G195000 (1) ATGTGTGGTATTCTTGCTGTCTTGTTGCTCTGATGACTCTCAGCCAA
51 100
CDS_Glmyma11G170300 (51) AAGGGTTCGCGTCTTGAGCTTCTCGCAGATTGAAGCACCGTGGTCCTG
CDS_Glyma.11G171400 (51) AAGGGTTCGCGTCTTGAGCTTCTCGCAGATTGAAGCACCGTGGTCCTG
CDS_Glyma.18G061100 (51) AAGGGTTCGCGTCTTGAGCTTCTCGCAGATTGAAGCACCGTGGTCCTG
CDS_Glyma.02G228100 (51) AAGGGTTCGCGTCTTGAGCTTCTCGCAGATTGAAGCACCGTGGTCCTG
CDS_Glyma.14G195000 (51) AAGGGTTCGCGTCTTGAGCTTCTCGCAGATTGAAGCACCGTGGTCCTG
101 150
CDS_Glmyma11G170300 (101) ACTGGAGTGGGCTCCACCAATATGGTGA TAACTATTTGGCTCATCAAAGG
CDS_Glyma.11G171400 (101) ACTGGAGTGGGCTCCACCAATATGGTGA TAACTATTTGGCTCATCAAAGG
CDS_Glyma.18G061100 (101) ACTGGAGTGGGCTCCACCAATATGGTGA TAACTATTTGGCTCATCAAAGG
CDS_Glyma.02G228100 (101) ACTGGAGTGGGCTCCATCAACATGGTGA CTGCTTTT TAGCATCAACGG
CDS_Glyma.14G195000 (101) ACTGGAGTGGGCTCCATCAACATGGTGA CTGCTTTT TAGCATCAACGG
151 200
CDS_Glmyma11G170300 (151) TTAGCCATAGTTGATCCAGGCTCTGGTGATCAACCCCTCTTCAATGAAGA
CDS_Glyma.11G171400 (151) TTAGCCATAGTTGATCCAGGCTCTGGTGATCAACCCCTCTTCAATGAAGA
CDS_Glyma.18G061100 (151) TTAGCCATAGTTGATCCAGGCTCTGGTGATCAACCCCTCTTCAATGAAGA
CDS_Glyma.02G228100 (151) TTAGCCATAGTTGATCCCTGCTCTGGTGATCAACCTCTTTTAAAGGGA
CDS_Glyma.14G195000 (151) TTAGCCATAGTTGATCCCTGCTCTGGTGATCAACCTCTTTTAAAGGGA
201 250
CDS_Glmyma11G170300 (201) CAAAACGTGTAGTGGTTACGGTGAATGGAGAGATCTACAATCATGAAGAAC
CDS_Glyma.11G171400 (201) CAAAACGTGTAGTGGTTACGGTGAATGGAGAGATCTACAATCATGAAGAAC
CDS_Glyma.18G061100 (201) CAAAACGTGTAGTGGTTACGGTGAATGGAGAGATCTACAATCATGAAGAAC
CDS_Glyma.02G228100 (201) CAAATCCGTCATTTGTCACGGTGAATGGAGAGATTTACAACCATGAAGAGC
CDS_Glyma.14G195000 (201) CAAATCCGTCATTTGTTACGGTGAATGGAGAGATTTACAACCATGAAGAGC
251 300
CDS_Glmyma11G170300 (251) TCAGGAAACAGTGCCTAATCACACCTTCCGTACAGGAAGTGACTGTGAT
CDS_Glyma.11G171400 (251) TCAGGAAACAGTGCCTAATCACACCTTCCGTACAGGAAGTGACTGTGAT
CDS_Glyma.18G061100 (251) TCAGGAAACAGTGCCTAATCACACCTTCCGTACAGGAAGTGACTGTGAT
CDS_Glyma.02G228100 (251) TCAGGAAACAGTGCCTAATCACAGTCCGTACTGGATGTGATGTGAT
CDS_Glyma.14G195000 (251) TCAGGAAACAGTGCCTAATCACACCTTCCGAAC TGGAAAGTGATGTGAT
301 350
CDS_Glmyma11G170300 (301) GTTATTGCTCACCTGTATGAGGACACGGAGAAAACCTTTGTGGACATGCT
CDS_Glyma.11G171400 (301) GTTATTGCTCACCTGTATGAGGACACGGAGAAAACCTTTGTGGACATGCT
CDS_Glyma.18G061100 (301) GTTATTGCTCACCTGTATGAGGACACGGAGAAAACCTTTGTGGACATGCT
CDS_Glyma.02G228100 (301) GTTATTGCTCACCTGTATGAGGACACGGAGAAAACCTTTGTGGACATGCT
CDS_Glyma.14G195000 (301) GTTATTGCTCACCTGTATGAGGACACGGAGAAAACCTTTGTGGACATGCT
351 400
CDS_Glmyma11G170300 (351) TGATGGTATATTTTCGTTTGTCTGCTAGATACTCGTGACAACAGTTTTTA
CDS_Glyma.11G171400 (351) TGATGGTATATTTTCGTTTGTCTGCTAGATACTCGTGACAACAGTTTTTA
CDS_Glyma.18G061100 (351) TGATGGTATATTTTCATTTGTCTGCTGGATACTCGTGACAACAGTTTTTA
CDS_Glyma.02G228100 (351) GGATGGTATCTTCATTTGTCTACTGGACACCCGTGACAACAGTTTTTA
CDS_Glyma.14G195000 (351) GGATGGTATCTTCATTTGTCTACTGGACACCCGTGACAACAGTTTTTA
401 450
CDS_Glmyma11G170300 (401) TAGTGGCACAAGATGCAATTGGGGTCACTTCCTTGTACATTGGTTGGGGT
CDS_Glyma.11G171400 (401) TAGTGGCACAAGATGCAATTGGGGTCACTTCCTTGTACATTGGTTGGGGT
CDS_Glyma.18G061100 (401) TAGTGGCACAAGATGCAATTGGGGTCACTTCCTTGTACATTGGTTGGGGT
CDS_Glyma.02G228100 (401) TAGTGGCTCGGGATGCTATTGGGGTCACTTCCTTGTACATTGGATGGGGA
CDS_Glyma.14G195000 (401) TAGTGGCTCGGGATGCTATTGGGGTCACTTCCTTGTACATTGGATGGGGG
451 500
CDS_Glmyma11G170300 (451) CTAGATGGCTCTGTCTGGATTTCATCAGAAATGAAGGGGTGAATGATGA
CDS_Glyma.11G171400 (451) CTAGATGGCTCTGTCTGGATTTCATCAGAAATGAAGGGGTGAATGATGA
CDS_Glyma.18G061100 (451) CTAGATGGCTCTGTCTGGATTTCCTCTSAATGAAGGGGTGAATGATGA
CDS_Glyma.02G228100 (451) CTAGATGGCTCTGTTGGATTTCATCAGAAATGAAGGGCCGAATGATGA
CDS_Glyma.14G195000 (451) CTAGATGGCTCTGTTGGATTTCATCAGAAATGAAGGGCCGAATGATGA
501 550
CDS_Glmyma11G170300 (501) TTGCGAACATTTTGAGTCTTTCCACCTGGTCACTTGTACTCTAGCAAAG
CDS_Glyma.11G171400 (501) TTGCGAACATTTTGAGTCTTTCCACCTGGTCACTTGTACTCTAGCAAAG
CDS_Glyma.18G061100 (501) TTGCGAACATTTTGAGTCTTTCCACCTGGTCACTTGTACTCTAGCAAAG
CDS_Glyma.02G228100 (501) TTGTTGAACATTTTGAGTCTTTCCACCTGGTCACTTGTACTCTAGCAAAG
CDS_Glyma.14G195000 (501) TTGTTGAACATTTTGAGTCTTTCCACCTGGTCACTTGTACTCTAGCAAAG
551 600

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ANNEXES

CDS_Glmyma11G170300	(551)	AGAGAGCGTTTCGCAGATGGTACAATCCTCCATGGTTCTCTGAGGCTATT
CDS_Glyma.11G171400	(551)	AGAGAGCGTTTCGCAGATGGTACAATCCTCCATGGTTCTCTGAGGCTATT
CDS_Glyma.18G061100	(551)	AGAGAGCGTTTCGCAGATGGTACAATCCTCCATGGTTCTCTGAGGCTATT
CDS_Glyma.02G228100	(551)	AAAGAGGGTTTCGCAGATGGTACAATCCTCCTGGTTCTCTGAGGCTACT
CDS_Glyma.14G195000	(551)	AAAGAGGGTTTCGCAGATGGTACAATCCTCCTGGTTCTCTGAGGCTATT
		601 650
CDS_Glmyma11G170300	(601)	CCCTCAGCACCTTATGATCCTCTTGCTTTGAGGCATGCCTTTGAGAAAGGC
CDS_Glyma.11G171400	(601)	CCCTCAGCACCTTATGATCCTCTTGCTTTGAGGCATGCCTTTGAGAAAGGC
CDS_Glyma.18G061100	(601)	CCATCTGCCCTTATGATCCTCTTGCTTTGAGGCATGCCTTTGAGAAAGGC
CDS_Glyma.02G228100	(601)	CCATCCACCCTTATGATCCTCTTGCTTTAAGACACACCTTTGAGCAGGC
CDS_Glyma.14G195000	(601)	CCATCTGCCCTTATGATCCTCTTGCTTTAAGACACGCCTTTGAGCAGGC
		651 700
CDS_Glmyma11G170300	(651)	TGTGGTAAAAAGTTGATGACTGATGTTCCCTTTGGTGTTTTGCTCTCTG
CDS_Glyma.11G171400	(651)	TGTGGTAAAAAGTTGATGACTGATGTTCCCTTTGGTGTTTTGCTCTCTG
CDS_Glyma.18G061100	(651)	TGTGGTAAAAAGTTGATGACTGATGTTGCCCTTTGGTGTTTTGCTCTCTG
CDS_Glyma.02G228100	(651)	AGTCAAAAAAGATTGATGACTGATGTTGCCCTTTGGTGTTCCTACTCTCTG
CDS_Glyma.14G195000	(651)	AGTCAAAAAAGTTGATGACTGATGTTGCCCTTTGGTGTTCCTACTCTCTG
		701 750
CDS_Glmyma11G170300	(701)	GAGGTTTGGACTCTTCATTGGTTGCAGCCGTCACGGCTCGTACC
CDS_Glyma.11G171400	(701)	GAGGTTTGGACTCTTCATTGGTTGCAGCCGTCACGGCTCGTACC
CDS_Glyma.18G061100	(701)	GAGGTTTGGACTCTTCATTGGTTGCAGCCGTCACGGCTCGTACC
CDS_Glyma.02G228100	(701)	GAGGTTTGGACTCTTCATTGGTTGCATCCGTCACCTCTCGTACC
CDS_Glyma.14G195000	(701)	GAGGTTTGGACTCTTCATTGGTTGCATCCATCACCTCTCGTACC
		751 800
CDS_Glmyma11G170300	(751)	GGCACAAATGCTGCAAGCAATGGGGAAACCAAATTACACTCTTTCTGTGT
CDS_Glyma.11G171400	(751)	GGCACAAATGCTGCAAGCAATGGGGAAACCAAATTACACTCTTTCTGTGT
CDS_Glyma.18G061100	(751)	GGCACAAATGCTGCAAGCAATGGGGAACTAAATTACACTCTTTCTGTGT
CDS_Glyma.02G228100	(751)	AAACACAAAGCTGCTGAGCAGTGGGGATCAAAGTTACATTCATTCTGTGT
CDS_Glyma.14G195000	(751)	AAACACAAAGCTGCTGAGCAGTGGGGATCAAAGTTACATTCATTCTGTGT
		801 850
CDS_Glmyma11G170300	(801)	AGGCCTTGAGGGTGCACCTGACCTAAAGGAGCAAAGGAAGTAGCAGACT
CDS_Glyma.11G171400	(801)	AGGCCTTGAGGGTGCACCTGACCTAAAGGAGCAAAGGAAGTAGCAGACT
CDS_Glyma.18G061100	(801)	AGGCCTTGAGGGTGCACCTGACCTAAAGGAGCAAAGGAAGTAGCAGAGT
CDS_Glyma.02G228100	(801)	AGGCCTTGAGGGCTCACCAGATTGAAAGGTCGAAAAGAAGTTGCTGACT
CDS_Glyma.14G195000	(801)	AGGCCTTGAGGGCTCACCAGATTGAAAGGTCGAAAAGAAGTTGCTGACT
		851 900
CDS_Glmyma11G170300	(851)	ACATAGGTAAGTACTGTACATCATGAATTTCACTACACTGTTCCAGGATGGCATA
CDS_Glyma.11G171400	(851)	ACATAGGTAAGTACTGTACATCATGAATTTCACTACACTGTTCCAGGATGGCATA
CDS_Glyma.18G061100	(851)	ACATAGGTAAGTACTGTCCATCATGAATTTCACTACACTGTTCCAGGATGGCATA
CDS_Glyma.02G228100	(851)	ATCTAGGCACTGTCCACCATGAGTTTACCTTCACTGTTCCAGGATGGATA
CDS_Glyma.14G195000	(851)	ATCTAGGCACTGTCCACCATGAGTTTACCTTCACTGTTCCAGGATGGATA
		901 950
CDS_Glmyma11G170300	(901)	GATGCCATTGAGGATGTGATCTATCACATTGAAACATATGATGTGACAAC
CDS_Glyma.11G171400	(901)	GATGCCATTGAGGATGTGATCTATCACATTGAAACATATGATGTGACAAC
CDS_Glyma.18G061100	(901)	GATGCCATTGAAAGATGTGATCTATCACATTGAGACATATGATGTGACAAC
CDS_Glyma.02G228100	(901)	GATGCCATTGAAAGATGTGATCTATCATGTTGAAACATATGATGTGACTAC
CDS_Glyma.14G195000	(901)	GATGCCATTGAAAGATGTGATCTATCATATTGAAACATATGATGTGACTAC
		951 1000
CDS_Glmyma11G170300	(951)	AATTAGAGCAAGCATTCCCATGTTTCTTATGTCTCGTAAGATCAAGTCAT
CDS_Glyma.11G171400	(951)	AATTAGAGCAAGCATTCCCATGTTTCTTATGTCTCGTAAGATCAAGTCAT
CDS_Glyma.18G061100	(951)	AATTAGAGCAAGCATTCCCATGTTTCTTATGTCTCGTAAGATCAAGTCAT
CDS_Glyma.02G228100	(951)	AATTAGAGGTAGCACACCATGTTTCTTATGTCTCGTAAGATTAATCAC
CDS_Glyma.14G195000	(951)	AATTAGAGCAAGCACCTATGTTTCTTATGTCTCGTAAGATTAATCAC
		1001 1050
CDS_Glmyma11G170300	(1001)	TGGGAGTCAAATGGTTTATATCTGGAGAAGGATCTGATGAGATCTTTGGA
CDS_Glyma.11G171400	(1001)	TGGGAGTCAAATGGTTTATATCTGGAGAAGGATCTGATGAGATCTTTGGA
CDS_Glyma.18G061100	(1001)	TGGGAGTCAAATGGTTTATCTCTGGAGAAGGATCTGATGAGATCTTTGGA
CDS_Glyma.02G228100	(1001)	TTGGTGTCAAATGGTTTATCTCG-AGAAGGATCTGATGAGATCTTTGGA
CDS_Glyma.14G195000	(1001)	TTGGTGTCAAATGGTTTATCTCAGGAGAAGGATCTGATGAGATCTTTGGA
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CDS_Glmyma11G170300	(1051)	GGGTATCTATATTTCCACAAGGCACCAACAAAGAAGAATTTCAATCAAGA
CDS_Glyma.11G171400	(1051)	GGGTATCTATATTTCCACAAGGCACCAACAAAGAAGATTTCAATCAAGA
CDS_Glyma.18G061100	(1051)	GGGTATCTATATTTCCACAAGGCACCAACAAAGAAGATTTCAATCAAGA
CDS_Glyma.02G228100	(1050)	GGGTATCTGTACTTTCCACAAGGCACCAACAAAGGAGGATTTCAATCAAGA
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CDS_Glyma.11G171400	(1101)	AACATGCCGCAAGATTAAAGCACTCCACAATATGATTGCTTGCGAGCCA
CDS_Glyma.18G061100	(1101)	AACATGCCGCAAGATTAAAGCACTCCACAATATGATTGCTTGCGAGCCA
CDS_Glyma.02G228100	(1100)	AACATGCCGCAAGATCAAAGCACTCCACAATATGATTGCTTGCGAGCCA
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CDS_Glyma.18G061100	(1151)	ATAAATCGACCTTTGCTGGGGTCTAGAAGCCAGAGTACCATTTTTGGAC		
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		1201		1250
CDS_Glmyma11G170300	(1201)	AAAGATTTTATCAGAGTGTGCAATGAACATTGATCCTGAGTATAAAAATGAT		
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CDS_Glyma.02G228100	(1200)	AAGGCATTTATCAATACGTGCAATGAGTATTGACCTGAGTCGAAAATGAT		
CDS_Glyma.14G195000	(1201)	AAGGCGTTTATCAATCGTGTGCAATGAGTATTGACCTGAGTGGAAATGAT		
		1251		1300
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		1301		1350
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CDS_Glyma.18G061100	(1301)	ATGATGAAGAACATCCTTATCTGCCAAAGCACATTTTATACAGGCAGAAA		
CDS_Glyma.02G228100	(1300)	ATGATGAAGAACATCCTTATCTGCCAAAGCACATTTTATACAGGCAGAAA		
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		1351		1400
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CDS_Glyma.02G228100	(1350)	GAGCAATTCAGTGATGGAGTTGGCTATAGCTGGATTGATGGCCTTAAAGC		
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CDS_Glmyma11G170300	(1401)	TCATGCTGAGAAACATGTGACTGATAGAATGATGCTCAATGCTGCTAACA		
CDS_Glyma.11G171400	(1401)	TCATGCTGAGAAACATGTGACTGACAGAATGATGCTCAATGCTGCTAACA		
CDS_Glyma.18G061100	(1401)	TCATGCTGAGAAACATGTAACTGACAGAATGATGCTCAATGCTGCTAACA		
CDS_Glyma.02G228100	(1400)	CCATGCTGCAGAAACATGTGACTGACAAAATGATGCTTAATGCTGCTAACA		
CDS_Glyma.14G195000	(1401)	CCATGCTGCAGAAACATGTGACTGAAAATGATGCTTAATGCTGCTAACA		
		1451		1500
CDS_Glmyma11G170300	(1451)	TTTTCCCCTTCAACACAACCAACCAAGAACATACACTATAAGAATG		
CDS_Glyma.11G171400	(1451)	TTTTCCCCTTCAACACAACCAACCAAGAACATACACTATAAGAATG		
CDS_Glyma.18G061100	(1451)	TTTTCCCCTTCAACACTCCAACCAAGAACATACACTATAAGAATG		
CDS_Glyma.02G228100	(1450)	TCTACCCCTACAACACCCCAACCAAGAACATATACTACAGAATG		
CDS_Glyma.14G195000	(1451)	TTTACCCCTACAACACCCCAAAACCAAGAACATATACTACAGAATG		
		1501		1550
CDS_Glmyma11G170300	(1501)	ATATTTGAGAGGTTCTTCCCTCAGAACTCAGCCAGGCTGAGTTTCCTGG		
CDS_Glyma.11G171400	(1501)	ATATTTGAGAGGTTCTTCCCTCAGAACTCAGCCAGGCTGAGTTTCCTGG		
CDS_Glyma.18G061100	(1501)	ATATTTGAGAGGTTCTTCCCTCAGAACTCAGCAAGGCTCACTGTTCTGG		
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		1551		1600
CDS_Glmyma11G170300	(1551)	AGGACCAAGTGTTCATGTAGCACAGCCAAAGCTGTTGAGTGGGATGCTG		
CDS_Glyma.11G171400	(1551)	AGGACCAAGTGTTCATGTAGCACAGCCAAAGCTGTAGAGTGGGATGCTG		
CDS_Glyma.18G061100	(1551)	AGGACCAAGTGTTCATGTAGCACAGCAAAAGCTGTTGAGTGGGATGCTG		
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		1601		1650
CDS_Glmyma11G170300	(1601)	CTTGGTCTAACAACTTGATCCATCTGGTAGAGCAGCACTTGGAGTGCAT		
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		1651		1700
CDS_Glmyma11G170300	(1651)	GCATCAGCTTATGGAATCAGGTCAA-----GCTGTAGAACGAGAGAA		
CDS_Glyma.11G171400	(1651)	GCATCAGCTTATGGAATCAGGTCAA-----GCTGTAGAACGAGAGAA		
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		1701		1750
CDS_Glmyma11G170300	(1695)	GATCATACCAAAGATGGAAGTTTCTCCACTAGGAGTTGCCATATAG----		
CDS_Glyma.11G171400	(1695)	GATCATACCAAAGATGGAAGTTTCCCCTAGGAGTTGCCATATAG----		
CDS_Glyma.18G061100	(1695)	GATCATACCAAAGATGGAAGTTTCTCCACTAGGAGTTGCCATATAG----		
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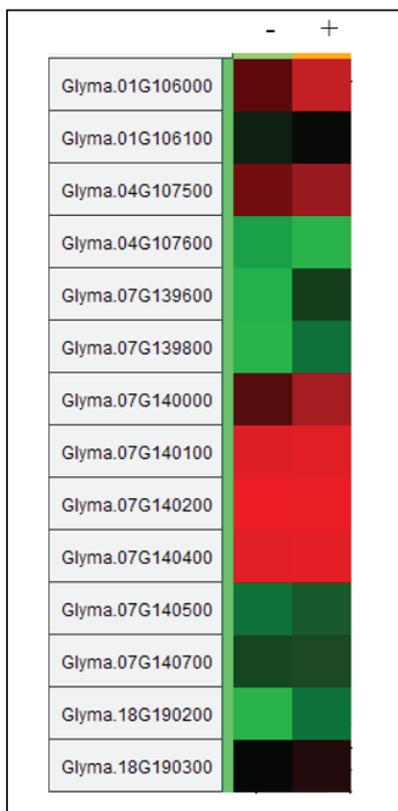
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#### Annex 4: sequence of the *GmCHIT1* promoter

ggcaagtgtgaaagaaccaactccaaaatggtatggaattggacttggagccatcatcatggtattgttcatcttcgtgctgtctctctc  
 ccacagtcctttactgaactgatgacttatccctactctttttgaggtttgcgtttgccgcaacaaagcggggtccatgatccaattccaat  
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 gg

Annex 5: **Gene expression of *gst1* soybean homologues *P. pachyrhizi* inoculation.** Mock (-) and *P. pachyrhizi* (+) inoculation at 24 h. Green: the lowest expression, red the highest and grey for no detection of expression. Internal RNA sequencing data from Bayer. Analysis performed with Gene data software.



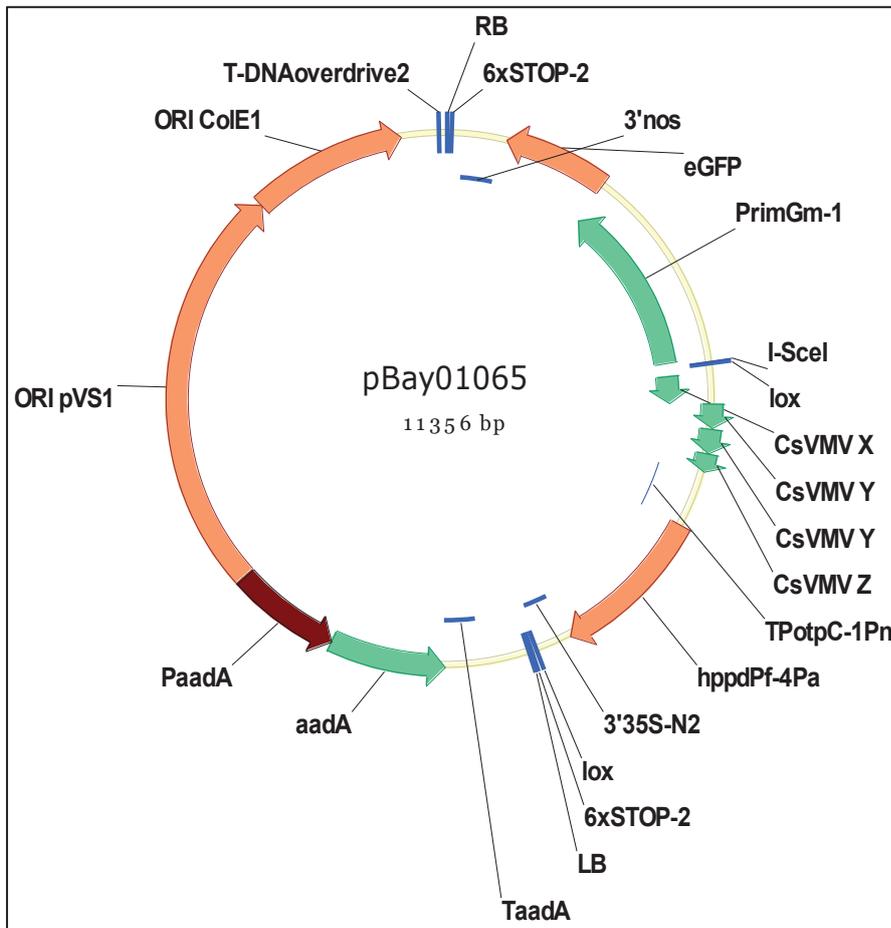
Annex 6: **sequence of the *gst1* promoter**

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 atttatactaaaaacatgtcacatgaatattgaaattataaaattatcaaaaataaaaaagaatatttcttaacaaattaa

ttgaaaatgatataaataaactattctatcattgattttctagccaccagatttgaccaaacagtgggtgacatgagcacata  
agtca

**Annex 7: sequence of the *GmRIM* promoter**

aaattatagtgaaaaaattctactttcaaaatttaagttaaagattctcaaaggaccatttaattaaagtataatattaattttt  
aatcaaatatataattatgtccatgttattttaattgttgatccacttataattttaagaaacttaaaatattgtaataaaatgcat  
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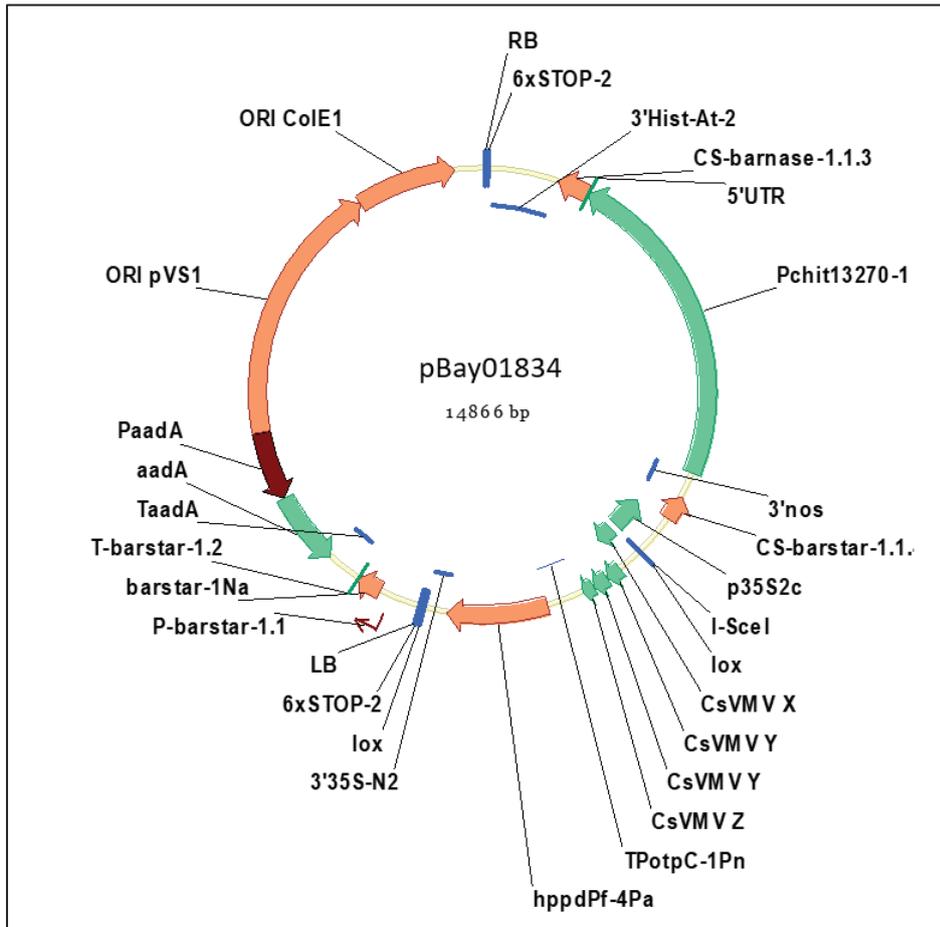
Annex 8: **pBay01065** plasmidAnnex 9: **sequence of the 35S promoter**

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aaggtaatatcgggaaacctcctcgattccattgccagctatctgtcacttcatcaaaaggacagtagaaaaggaaggtggcacct  
acaaatgccatcattgcgataaaggaaaggctatcgttcaagatgccctgccgacagtggtccaaagatggacccccaccacga  
ggagcatcgtggaaaaagaagacgtccaaccacgtcttcaagcaagtggattgatgtgatattccactgacgtaagggatgacgc  
acaatcccactatccttcgaagaccttctctatataaggaagttcatttcatttgagagga

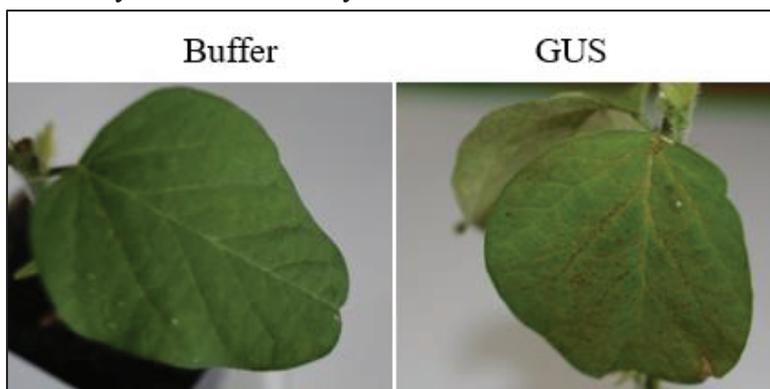
Annex 10: **sequence of the NOS promoter**

Atccggtgcagattatttgattgagagtgaatatgagacttaattggataccgaggggaatttatggaacgtcagtgaggcatttt  
tgacaagaaatatttgctagctgatagtgaccttaggcgactttgaacgcgcaataatggtttctgacgtatgtgcttagctcattaa  
actccagaaaccgcggctgagtggtccttcaacgttgcggttctgtcagttcacaacgtaaacggcttcccgcgtcatcggcg  
ggggtcataacgtgactcccttaattctccgctca

## Annex 11: pBay01834 plasmid



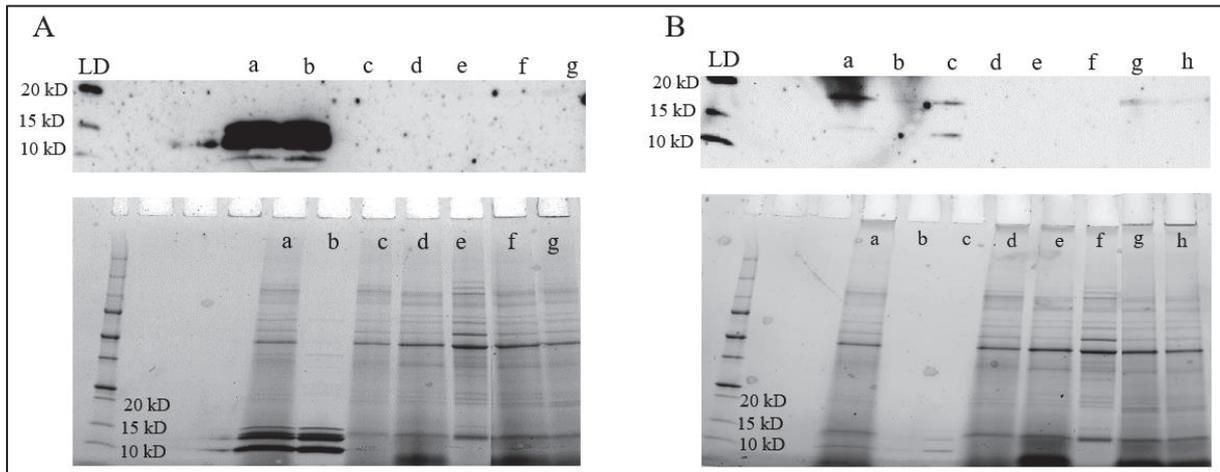
Annex 12: Transient expression in soybean of a construct expressing the GUS gene. Images of the soybean leaves 2 days after transient transformation.



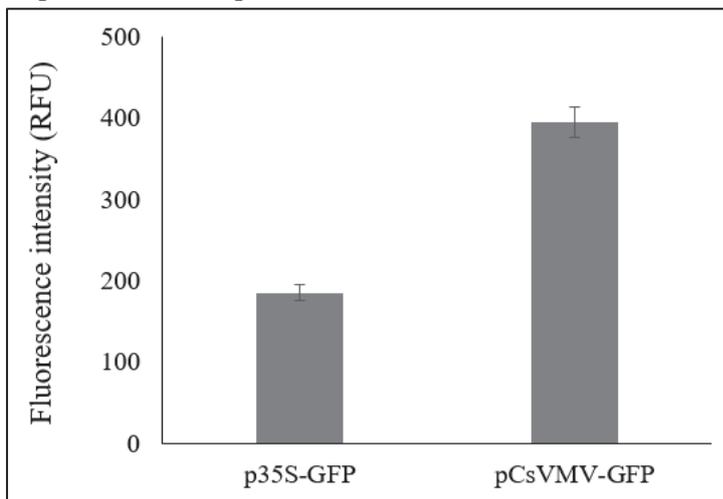
**Annex 13: Verification of transgene insertion on the T0 events obtained after soybean transformation with the pBay01404 plasmid.**

	Samples	Barnase	Barstar	HPPD	Sequence outside the LB/RB border
T0 events	01	Yes	Yes	Yes	Yes
	02	Yes	Yes	Yes	Yes
	03	Yes	Yes	Yes	No
	04	Yes	Yes	Yes	No
	05	Yes	Yes	Yes	No
	06	Yes	Yes	Yes	No
	07	Yes	Yes	Yes	Yes
	08	Yes	Yes	Yes	Yes
	09	Yes	Yes	Yes	Yes
	10	Yes	Yes	Yes	No
	11	Yes	Yes	Yes	No
	12	Yes	Yes	Yes	No
	13	Yes	Yes	Yes	No
	14	Yes	Yes	Yes	No
	15	Yes	Yes	Yes	No
	16	Yes	Yes	Yes	No
	17	Yes	Yes	Yes	No
	18	Yes	Yes	Yes	Yes
	19	Yes	Yes	Yes	Yes
	20	Yes	Yes	Yes	No
	21	Yes	Yes	Yes	Yes
	22	Yes	Yes	Yes	No
	23	Yes	Yes	Yes	Yes
	24	Yes	Yes	Yes	Yes
	25	No	No	Yes	Yes
Positive control: plasmid pBay01404		Yes	Yes	Yes	Yes
Negative control: WT plant		No	No	No	No

**Annex 14: Detection of barnase and barstar proteins.** Barnase expectide size: 12kDa and barstar expectide size 10kDa. A) barnase detection 2 days post transient transformation of diferent constructs in *N. benthamiana*. a) barnase protein (synthetis by an external laboratory, 300 mg) and tobacco proteins (50 mg), b) barnase protein, 300 mg, c) WT tissues, d) GFP plasmid, e) pNOS:barstar, f) pBay01842 and g) pBay01834 constrcuts. B) barstar detection 2 days post transient transformation of diferent constructs in *N. benthamiana* (same samples than in A). a) barstar protein (synthetis by an external laboratory, 300 mg) and tobacco proteins (50 mg), b) barstar protein (50 mg), c) barstar protein (300 mg), d) WT tissues, e) GFP plasmid, f) pNOS:barstar, g) pBay01842 and h) pBay01834 constructs.



**Annex 15: GFP expression under the control of *CsVMV* or 35S promoters after transient expression in *N. benthamiana*.** Observations were performed 4 days post-agro-infiltration. a) Fluorescence intensity in relative fluorescence units (RFU) measured after transient expression of p35S:GFP and p*GmCHIT1*:GFP constructs. Mean of 12 replicates  $\pm$  SD.





# LEXIQUE

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- ABA: Abscisic Acid
- ABC: ATP-binding cassette
- ACC: 1-aminocyclopropane-1-carboxylic acid
- AFLP: Amplified fragment-length polymorphism
- ATP: Adenosine Triphosphate
- AUX: Auxin
- Avr: Avirulence protein
- BAP: 6-benzylaminopurine
- BR: Brassinosteroid
- *Bt*: *Bacillus thuringiensis*
- CaMV: Cauliflower mosaic virus
- CCM: Co-culture medium
- cDNA: complementary DNA
- CERK1: Chitin Elicitor Kinase
- CK: Cytokinin
- CsVMV: Cassava vein mosaic virus
- CWP: Cell wall deposition
- DAMP: Damage associated molecular pattern
- ddPCR: digital droplet PCR
- DMI: Demethylation inhibitors
- dpi: day post infection
- DP7: chitinheptaose
- DTT: Dithiothreitol
- EDS1: Enhanced disease susceptibility 1
- EDTA: Ethylenediaminetetraacetic acid
- ETI: Effector triggered immunity
- ETS: Effector triggered susceptibility
- FAO: Food and agriculture organization
- flg22: 22 amino acid epitope of the bacterial flagelin N-terminus
- FLS2: Flagelin Sensing 2
- FPKM: Fragments per kilobase per million of reads
- GA: Gibberellin acid
- GA3: gibberellin acid A3

- GFP: Green Fluorescence protein
- GM: Genetically modified
- GST: Glutathione-S-transferase
- GUS:  $\beta$ -glucuronidase
- HIGS: Host-induced gene silencing
- HLB: Citrus huanglongding
- hpi: hours post infection
- HPPD: Hydroxyphenylpuruvate dioxygenase
- HR: Hypersensitive response
- IAA: indole-3-acetic acid
- IM: Immune response
- JA: Jasmonic Acid
- LB: Left border
- Lb: Lysogeny broth medium
- LRR: Leucine-riche repeat
- MAMP: Microbial associated molecular pattern
- MAPK: Mitogen activated protein pinase
- MES: 2-(*N*-morpholino)ethanesulfonic acid
- miRNA: micro-RNA
- mRNA: messenger RNA
- MS: Murashige and Skoog salt
- NBS: Nucleotide biding site
- NGS: Next generation sequencing
- NHR: Non-host resistance
- NLR: Nucleotide-binding leucine-rich repeat
- NO: Nitric Oxide
- NOS: Nopaline synthase
- OMR: Overlapping motif region
- PAD4: Phytoalexine-deficient 4
- PAMP: Pathogen Associated Molecular Pattern
- PCD: Programed cell death
- PCR: polymerase chain reaction
- PEN: Penetration

- PG: Polygalacturonase
- PGIP: Polygalacturonase-inhibiting protein
- PpEC: *P. pachyrhizi* effector candidate
- PR: Pathogenesis related protein
- PRR: Pattern recognition receptor
- PTI: PAMP triggered immunity
- PVY: Potato virus Y
- Qoi : Quinone outside inhibitor
- qPCR: quantitative PCR
- QTL: Quantitative trait loci
- R: Resistance
- RB: Red brown lesion
- RNAi: RNA interference
- RNase: Ribonuclease
- RNA-seq: RNA sequencing
- ROS: Reactive oxygen species
- Rpp: Resistance to *P.pachyrhizi*
- RR: Roundup ready
- RT: Reverse transcription
- S: susceptibility
- SA: Salicylic Acid
- SAG101: Senescence-associated gene 101
- SAR: Systemic acquire resistance
- SDHI: Succinate dehydrogenase inhibitors
- SE: Shoot elongation medium
- SI: Shoot initiation medium
- siRNA: small interfering RNA
- sRNA: small non-coding RNA
- TAN: tan colored lesion
- TBT: Tembotrione
- TEV: tobacco etch virus
- TF: Transcription factor
- TSS: Transcription start site

## LEXIQUE

- VIGS: Virus induced gene silencing
- *vir*: virulence gene
- WT: Wild Type



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