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Suppression de la réponse immunitaire des racines d'*Arabidopsis thaliana* par la phytotoxine coronatine produite par *Pseudomonas syringae*
&
Étude fonctionnelle du cytochrome P450 CYP76C2 d'*Arabidopsis thaliana* impliqué dans la réponse aux pathogènes

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Suppression of *Arabidopsis thaliana*

root innate immunity by *Pseudomonas syringae*

phytotoxin coronatine

&

Functional studies of the cytochrome P450 CYP76C2

a thesis presented by

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Abbreviations

ABA: abscisic acid
ACC: 1-aminocyclopropane-1-carboxylic acid
BFA: brefeldin A
CFA: coronafacic acid
CMA: coronamic acid
COR: coronatine
CWDE: cell wall degrading enzyme
DAMP: danger-associated molecular pattern
EMS: ethyl methane sulfonate
ET: ethylene
ETI: effector-triggered immunity
EZ: elongation zone
FB1: fumonisin B1
GUS: β-glucuronidase
HR: hypersensitive response
I3G: indol-3-ylmethylglucosinolate
ISR: induced systemic resistance
JA: jasmonate
LPS: lipopolysaccharide
LRR: leucine rich repeat
RLK: receptor like kinase
MAMP: microbe-associated molecular pattern
MAPK: mitogen activated protein kinase
NBS-LRR: nucleotide binding site-leucine rich repeat receptor
OG: oligogalacturonides
PAMP: pathogen-associated molecular pattern
PGN: peptidoglycan
PGPR: plant growth promoting rhizobacteria
PID: pre-invasion defense
PRR: pathogen recognition receptor
PTI: PAMP-triggered innate immunity
R-gene: resistance gene
RLK: receptor like kinase
ROS: reactive oxygen species
R-protein: resistance protein
SA: salicylic acid
SAR: systemic acquired resistance
SGM: seedling growth media
T3E: type III effector
TLR: toll like receptor
TTSS: type III secretion system
X-gluc: 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid

Résumé

Au cours de l'évolution, les plantes ont développé des mécanismes de défense sophistiqués contre les pathogènes. L'une des premières lignes de défense se base sur la reconnaissance par la plante de motifs moléculaires très conservés associés aux pathogènes (PAMP/MAMP). Cette reconnaissance active divers mécanismes de défense, en particulier le dépôt de callose au niveau de la zone infectée. Malgré l'abondance des interactions racine-microbes, la réponse aux MAMPs dans cette partie de la plante reste largement inexplorée. Nous avons développé un système de culture hydroponique qui nous a permis d'étudier cette réponse chez *Arabidopsis thaliana* en se basant sur l'étude de lignées *promoteur:GUS* ainsi que sur le dépôt de callose Nous avons trouvé que les racines répondent fortement aux MAMPs dans des régions bien spécifiques, en particulier dans la zone d'élongation. Cette réponse dépend de la voie de signalisation de l'éthylène, du facteur de transcription MYB51, du cytochrome P450 CYP81F2 ainsi que de la myrosinase PEN2. En outres, nous montrons que *Pseudomonas syringae* et *Pseudomonas fluorescens* sont capables de bloquer ce mécanisme de défense. En particulier, dans le cas de *P. syringae*, cette suppression s'effectue grâce à la production de coronatine (COR). L'action de la COR est dépendante de l'E3 ligase COI1 et du facteur de transcription JIN1/MYC2. Un screen génétique m'a permis d'isoler de nouveaux mutants incapables de bloquer la réponse aux MAMPs, dans le but d'identifier de nouveaux gènes impliqués dans la réponse à la COR. Enfin, ma thèse a porté sur l'étude du cytochrome P450 CYP76C2, fortement induit par les pathogènes. CYP76C2 est activé localement lors d'une infection par *P. syringae* ou *Botrytis cinerea* ainsi que lors des mécanismes de mort cellulaire. Je démontre que l'activation de CYP76C2 est partiellement dépendante de la voie de signalisation de l'acide salicylique et que ce gène est potentiellement impliqué dans le contrôle du stress oxydatif.

Mots clés: plante, racines, pathogènes, PAMP, callose, coronatine, cytochrome P450, stress oxydatif.

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Abstract

Over the course of evolution, plants developed sophisticated defense mechanisms against bacterial and fungal pathogens. One of the first layers of plant defense is called PAMP triggered immunity (PTI) and is based on the recognition of conserved epitopes of pathogen-derived molecules called PAMPs/MAMPs (Pathogen/Microbe Associated Molecular Patterns). This recognition activates defense responses including the deposition of callose at the site of pathogen attack. Despite the fact that roots are the organs most subject to microbial interactions, MAMP signaling in roots remains largely unexplored. I developed an *Arabidopsis thaliana* seedling assay to study PTI in roots based on the detection of callose and the activation of *promoter:GUS* reporters of MAMP-responsive genes. I found that MAMPs trigger a strong response in roots dependent on ethylene signaling, the MYB51 transcription factor, the cytochrome P450 CYP81F2, and the PEN2 myrosinase, but independent of salicylic acid signaling. In addition, I show that the bacteria *Pseudomonas syringae* and *Pseudomonas fluorescens* suppress this response and that *P. syringae* is doing so by producing the phytotoxin coronatine. I found that coronatine acts via the E3 ligase COI1 and the transcription factor JIN1/MYC2. I performed a forward genetic screen to isolate mutants impaired in COR-mediated suppression in an attempt to identify new players involved in COR signaling. In this thesis, I also present data concerning *CYP76C2*, a gene encoding a cytochrome P450 that is highly induced by MAMPs and pathogens in *Arabidopsis* leaves. I confirmed that *CYP76C2* is activated during pathogen infection and various cell death elicited scenarios. Furthermore, I demonstrate that CYP76C2 is partially dependent on SA signaling and may be involved in controlling oxidative damage during infection.

Keywords: plant, roots, pathogens, PAMP, callose, coronatine, cytochrome P450, oxidative stress.

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Chapter 1. Introduction

The constant increase of the world population, especially in developing countries, is raising concerns about the sufficiency of the food supply. Therefore, agriculture constantly needs to improve its productivity, sometimes at the expense of the protection and conservation of our environmental resources. A challenge agriculture is facing to improve its yields is pathogens (bacterial or fungal). A significant portion of major crops ranging from 3 to 33% is lost worldwide before and after harvest depending on the crop and where it is grown due to pathogen infections (Oerke, 2006). Some pathogens have caused some of the deadliest famines in history such as the great Irish famine (1845-1850) caused by the late blight of potato (*Phytophtora infestans*). Over the years, the war against pests and pathogens have led us to use more and more pesticides every year with deleterious consequences on the environment, in particular on the soil microflora, and on public health, especially among agricultural workers. This situation, coupled with the appearance of pathogens with increased resistance against pesticides, highlights the need for new strategies to contain pathogens. These include classical pathogen containment practices such as crop rotation, use of resistant varieties, biological control, and scouting programs for early detection, as well as the development of genetically modified crops etc. Understanding how plants defend themselves against pathogens, how pathogens successfully attack plants, and the ecological consequences of such interactions, will be key to improve existing strategies for pathogen control, developing new ones, and assessing their potential risks to the environment and public health.

I. The plant defense mechanisms against pathogens

Unlike most animals, plants are sessile, and have therefore developed very efficient mechanisms enabling them to adapt to various environmental stresses. One of the most important aspects of the ability of a plant to survive is its capacity to resist pathogen attacks. Despite the fact that plants are constantly challenged by microbes, only a small fraction of these attacks result in a successful infection. This is due to the highly sophisticated defense mechanisms that plants developed during evolution. This section of the Introduction briefly describes the multiple layers of immune responses that plants have developed.

The boy-scout motto, "be prepared", well describes the first layer of plant defense, called pre-invasion defense (PID). PID is conferred by constitutive physical and chemical barriers that prevent microbes from entering host tissues. One of the most important components of this defense is the cell wall. The many structural polymers constituting the cell wall such as lignin, cellulose or suberin provide the plant with an extremely robust and efficient physical barrier (Huckelhoven, 2007). Another line of defense is provided by the constitutive production of non-specific antimicrobial compounds. The secretion of these secondary metabolites in the apoplast creates a hostile environment for potential pathogens (Huckelhoven, 2007).

A second layer of plant defense is called PAMP-triggered immunity (PTI). Potential pathogens can be detected by pathogen recognition receptors (PRRs) recognizing conserved epitopes of pathogen-derived molecules called PAMPs (for Pathogen Associated Molecular Patterns) (Figure 1A) (Schwessinger and Zipfel, 2008). PAMPs are microbe-derived molecules such as flagellin, elongation factor Tu,

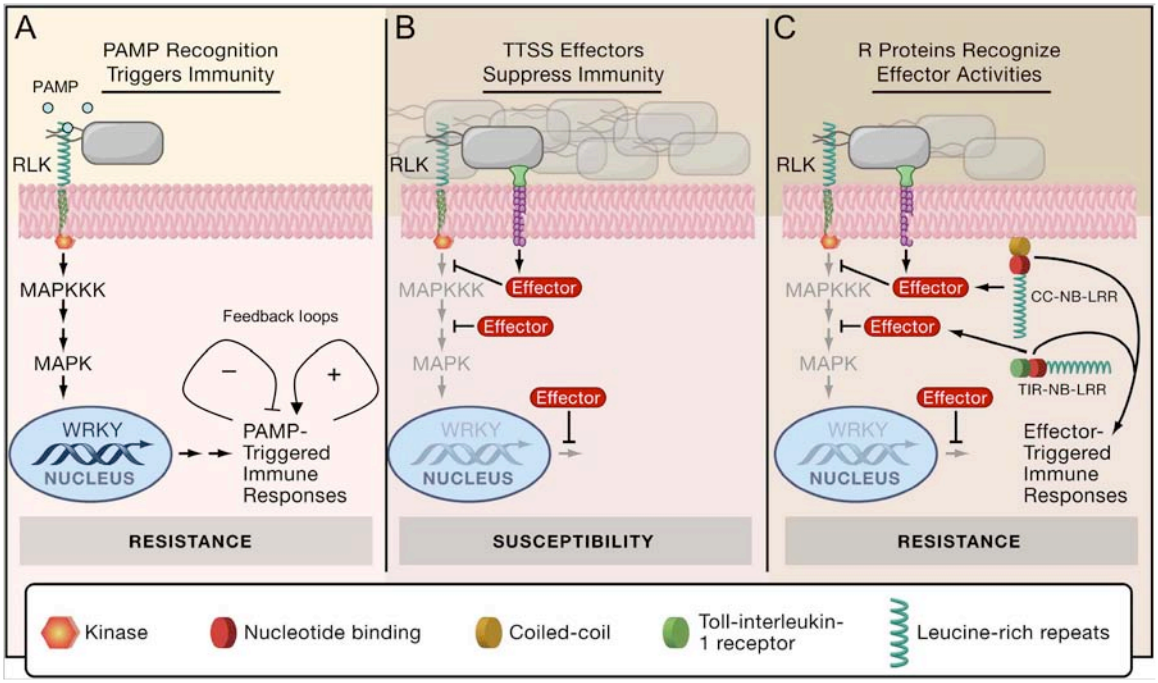

Figure 1. Model of plant innate immunity.
(A) Activation of PTI by recognition of PAMPs/MAMPs and/or DAMPs by PRRs during microbial infection. This activation is regulated by positive and/or negative feedback loops.
(B) Suppression of PTI by pathogen effectors.
(C) Activation of ETI by recognition of pathogen effectors.
Adapted from Chisholm et al., 2006.

peptidoglycan (PGN) or chitin. PAMPs do not necessarily come from pathogens and will therefore be designated as microbe associated molecular patterns (MAMPs) in this thesis. During PTI, plants can also recognize endogenous elicitors produced upon infection such as oligogalacturonides (OGs) (Hahn et al., 1981), sugar polymers released from the pectin component of the plant cell wall or the MAMP-inducible AtPep1, a small endogenous peptide synthesized in response to pathogens and recognized by the plant to amplify PTI through a positive feedback (Huffaker and Ryan, 2007; Yamaguchi et al., 2006). Molecules such as OGs and AtPep1 are referred as danger associated molecular patterns (DAMPs). The mechanisms responsible for the resistance against pathogens as a consequence of PTI are not well understood. PTI is described in more detail in the next section. PID and PTI are non-specific defense mechanisms generally sufficient to protect the host against potential pathogens and are usually referred to as "basal resistance".

Successful pathogens have evolved strategies to overcome this basal resistance by secreting proteinacious effectors into plant cells to suppress PTI and PID (Figure 1B) (Block et al., 2008). In return, plants developed resistance proteins (R-proteins), encoded by resistance genes (*R*-genes), that recognize pathogen-encoded effectors or detect the changes of the host targets induced by those effectors (the latter referred to as the "guard hypothesis") (Figure 1C) (Chisholm et al., 2006). Recognition of effectors or effector action by R-proteins leads to a strong and rapid defense response known as the hypersensitive response (HR). In particular, HR is characterized by programmed cell death (PCD) that restricts pathogen growth (Greenberg and Yao, 2004). This "scorched earth" strategy, based on the recognition of specific pathogen effectors, is referred as effector-triggered immunity (ETI), *R*-gene mediated immunity, or race-specific

resistance.

ETI also triggers the accumulation of salicylic acid (SA), a major plant defense hormone against biotrophic pathogens (Loake and Grant, 2007). SA signaling confers local resistance as well as an increased resistance in systemic tissue known as the systemic acquired resistance (SAR) (Vlot et al., 2008). The biggest class of plant *R*-genes is an extensive family of intracellular proteins containing a nucleotide-binding site and N-terminal leucine rich repeats (NBS-LRR) (DeYoung and Innes, 2006). A central component of ETI mediated by TIR-NBS-LRRs, a sub-class of the NBS-LRRs carrying an N-terminal TIR domain (for Toll Interleukine Receptor), is the protein EDS1 (Wiermer et al., 2005). *EDS1* is required for both HR and SA accumulation mediated by these receptors. In addition to ETI, *EDS1* has been shown to play an important role in basal resistance against biotrophic pathogens in the absence of *R*-gene mediated recognition of bacterial effectors. Indeed, *eds1* mutants are defective in basal resistance against virulent *Peronospora parasitica*, *Erysiphe,* and *Pseudomonas syringae*. In both cases, ETI and basal resistance, EDS1 and its interacting partner PAD4 are required for SA accumulation and SA-mediated resistance (Aarts et al., 1998; Parker et al., 1996; Xiao et al., 2005). EDS1 and PAD4 share some level of homology with eukaryotic lipases and could potentially metabolize lipids that play an important role in signaling. Unfortunately, no evidence has proven this hypothesis so far and the exact role of EDS1 and PAD4 in signaling is still not fully understood. However, increasing evidence suggest that EDS1 and PAD4 may be involved in transducing redox signaling (Wiermer et al., 2005).

In addition to SA, two additional plant hormones are involved in basal defense

against pathogens, jasmonate (JA) and ethylene (ET). Unlike SA signaling, conferring resistance against biotrophic pathogens, genetic studies have shown that JA and ET signaling are more effective against necrotrophic pathogens (Kunkel and Brooks, 2002). However, ET functions more like a modulator of JA and SA signaling than a defense hormone conferring resistance *per se*. Indeed, It was shown that ET potentiates SA signaling during an attack by a biotroph (De Vos et al., 2006; Lawton et al., 1994) and modulates crosstalk between SA and JA signaling pathways (Leon-Reyes et al., 2009). Finally, it is well known that many JA-dependent defense genes are also regulated by ET-signaling (Broekaert et al., 2006).

II. The PAMP-triggered immunity (PTI)

The first evidence that PRRs are an essential part of innate immunity in animals came from the discovery of the Toll receptor in Drosophila. It appeared that the *toll* mutant, initially found to show abnormal dorsal-ventral embryonic development, was also greatly impaired for its resistance against fungal pathogens (Lemaitre et al., 1996). This led to the discovery of the Toll-like receptors (TLRs) in mammals (Medzhitov et al., 1997). TLRs constitute a superfamily of transmembrane proteins sharing a high homology in their intracellular domain called the TIR domain (for Toll Interleukine Receptor). The extracellular domain of TLRs is composed of large LRR domains that bind directly or indirectly to a specific MAMP. MAMP recognition triggers the binding of intracellular adaptors to the TIR domain of TLRs and the subsequent activation of a signaling cascade leading to the activation of various transcription factors. This can induce the production of antimicrobials, signaling molecules such as cytokines and

Table 1. Non-exhaustive list of PAMP/MAMP/DAMPs in plant defense

PAMP/MAMP/DAMP	Epitope	Responsive plants	Receptor	References
Bacterial PAMP/MAMP				
Cold-shock protein	Csp15	Solanaceae	Unknown	{Felix, 2003}
Elongation factor Tu	Elf18	Brassicaceae	AtEFR (LRR-RLK)	{Kunze, 2004; Zipfel, 2006}
Flagellin	Flg22	Most plants	AtFLS2, LeFLS2, NbFLS2 (LRR-RLKs)	{Felix, 1999; Gomez-Gomez, 2000; Hann, 2007; Robatzek, 2007}
Harpin	Unknown	Various plants	Unknown	{He, 1993; Lee, 2001; Wei, 1992}
Lipopolysaccharides (LPS)	Unknown (lipid A?)	*Arabidopsis*, pepper, tobacco	Unknown	{Meyer, 2001; Newman, 1995}
Nod factors	Unknown	Legumes	NFR1, NFR5 (LysM-RLKs)	{Limpens, 2003; Radutoiu, 2003}
Peptidoglycan (PGN)	Unknown	*Arabidopsis*	Unknown	{Gust, 2007}
Fungal PAMP/MAMP				
β-glucans	Branched hepta-β-glucosides Linear oligo-β-glucosides	Legumes Tobacco	Unknown Unknown	{Fliegmann, 2004; Umemoto, 1997} {Klarzynski, 2000}
Cellulose binding elicitor lectin	Cellulose binding domain	Tobacco, *Arabidopsis*	Unknown	{Gaulin, 2006}
Chitin	Chitooligosaccharides polymers ≥ 4 residues	*Arabidopsis*, Tomato, rice, wheat	AtCERK1 (LysM-RLK)?	{Georg Felix, 1993; Miya, 2007}
Ergosterol	Unknown	Tomato	Unknown	{Granado, 1995}
Invertase	N-mannosylated peptide	Tomato	Unknown	{Basse, 1992; Basse, 1993}
Necrosis inducing proteins	Unknown	Several dicotyledones	Unknown	{Fellbrich, 2002; Veit, 2001}
Transglutaminase	Pep13	Parsley, potato	Unknown	{Brunner, 2002; Nurnberger, 1994}
Xylanase	TKLGE pentapeptide	Tobacco, tomato	LeEIX2 (LRR-RLP)	{Ron, 2004; Rotblat, 2002; Hanania, 1997}
DAMP				
Oligogalacturonides	9 ≤ Polymers ≤ 16 residues	Various plants	Unknown	{Hahn, 1981}
AtPep1	Unknown	*Arabidopsis*	AtPEPR1 (LRR-RLK)	{Huffaker, 2006; Yamaguchi, 2006}

chemokines, and an inflammatory reaction (O'Neill, 2008).

Plants have developed a similar system to recognize potential invaders. Various MAMPs can be recognized by plants. Pep13, a short peptide derived from a transglutaminase of *Phytophtora sojae,* was the first clearly defined MAMP (Nurnberger et al., 1994). The MAMPs described in plants so far come from bacteria or fungi. No MAMP from a virus has been identified so far. MAMPs from bacteria include Flg22, Elf18 (or Elf26) and csp22, small peptides respectively derived from the bacterial flagellin, the bacterial elongation factor Tu and the bacterial cold shock protein CSP. Lipopolysaccharides (LPS) and PGN are two other well-described bacterial MAMPs. The MAMPs from fungi include Pep13, cell-wall components such as chitin and β-glucan, and ergosterol. In addition to MAMPs, plants also recognize different DAMPs including OGs and AtPep peptides. Table 1 provides a non-exhaustive list of known MAMPs and DAMPs.

PRRs have been identified for only a small fraction of these MAMPs and are, in most cases, receptor-like kinases (RLKs). It was shown that *Arabidopsis* mutants corresponding to these PRRs are more susceptible to pathogens demonstrating the importance of PTI in plant defense (Zipfel et al., 2006; Zipfel et al., 2004). In *Arabidopsis*, only two PRRs have clearly been identified, the leucine-rich repeat (LRR) RLKs FLS2 and EFR, respectively binding to flagellin and EF-Tu. The *Arabidopsis* LysM-RLK CERK1 has been shown to be required for the response to chitin, suggesting that CERK1 is the PRR for chitin. However, it has not been demonstrated that CERK1 specifically binds to chitin. MAMP recognition triggers many responses including an oxidative burst mediated by the NADPH oxidase ATRBOHD, an increase in intracellular

Ca^{2+} concentration, and ethylene and nitric oxide production (Gomez-Gomez and Boller, 2002; Nuhse et al., 2007). The roles of those early events are still poorly understood but may play a role in signaling.

FLS2 is by far the best studied PRR in plants and its orthologs in tomato and tobacco have been identified (Hann and Rathjen, 2007; Robatzek et al., 2007). FLS2 has been shown to be internalized after binding to Flg22, similarly to what was found in mammals with TLRs (Robatzek et al., 2006). This endocytosis is dependent on its kinase activity and its PEST motif, believed to be ubiquitinated upon internalization. The role and mechanism of internalization is not well understood but is believed to be required for signaling. FLS2 has also been shown to bind to another LRR-RLK, BAK1, after binding to Flg22 (Chinchilla et al., 2007). This interaction is required for signaling but not for Flg22 binding, suggesting a role of BAK1 in transducing the signal.

MAMP recognition by PRRs activates a complex network of MAPKs (Figure 1A). This aspect of PTI is still not fully understood but some components have been identified. The MAPKKK AtMEKK1 was shown to initiate the Flg22 MAPK cascade and to activate the MAPKKs AtMKK4 and AtMKK5. Those two MAPKKs activate the MAPKs AtMPK3 and AtMPK6 leading to the activation early defense genes (Asai et al., 2002). However, we are far from completely understanding the various mechanisms taking place during this step of PTI, because even though several MAMPs appear to signal through this same cascade, the downstream gene activation patterns are MAMP-specific. Another aspect complicating the these studies is the involvement of these MAPKs in various processes other than PTI, including hormone signaling and the response to different abiotic stresses.

Downstream of the MAPKs network is the activation of a number of transcription factors including *WRKYs*, *MYBs*, *ERFs* (Figure 1A). The roles of those transcription factors is, for the most part unknown, but are believed to either control the expression of defense genes involved in PTI, or negatively regulate PTI itself. For example, *MYB51* has been shown to be required for callose deposition in cotyledons after Flg22 treatment and is a major regulator of indole glucosinolate biosynthesis (Clay et al., 2009; Gigolashvili et al., 2007). *WRKY11*, *17*, *18* and *40*, all activated by Flg22, were shown to be negative regulators of basal resistance suggesting a negative feedback regulation of PTI after MAMP signaling, probably to fine tune the response (Journot-Catalino et al., 2006; Xu et al., 2006). However, none of the mutants in these latter transcription factors are impaired for PTI.

It is still unclear what are the actual effectors leading to PTI. Callose deposition, a well-known response to MAMPs, is believed to play a role by strengthening the cell wall and preventing pathogen penetration, but can not account for the entire resistance triggered by MAMPs. For example, *Arabidopsis* mutants that cannot synthesize callose in response to MAMPs are only marginally more susceptible to *P. syringae* (Clay et al., 2009). Antimicrobial production after MAMP elicitation takes place in *Arabidopsis* seedlings as shown in unpublished work in the Ausubel lab by postdoctoral fellow Cristian Danna, however the identification of the antimicrobial compounds has not been determined. Finally, the level of SA has been shown to increase during PTI, and mutants in the SA signaling pathway are partially compromised for their PTI-induced defense (Mishina and Zeier, 2007; Tsuda et al., 2008). A lot of work still needs to be done to clarify the actual importance of those different responses for PTI and to understand their

regulation.

III. Virulence strategies of pathogens

Pathogens have evolved extremely diverse strategies to become successful invaders. Pathogens can secrete toxins, inject effectors into the plant cell to promote disease, secrete cell wall degrading enzymes or manipulate plant hormone signaling pathways to deregulate defense mechanisms. This section briefly describes examples illustrating these strategies.

Many pathogens secrete phytotoxins to increase their virulence. These low molecular weight toxins can directly damage host tissue or manipulate plant metabolism to facilitate the infection process. The two kind of lipodepsipeptide toxins produced by *P. syringae*, syringomycins and syringopeptins, are examples of the first class of toxins. They form small pores in the plasma membrane of host cells resulting in ion leakage and eventual to cell death (Bender et al., 1999). *P. syringae* is believed to benefit from the nutrients released from the plant cells during this process. An example of the second class of toxins is the chlorosis inducing phytotoxin coronatine (COR) that is also produced by *P. syringae* (Bender et al., 1999). COR acts as a mimic of JA (Feys et al., 1994; Weiler et al., 1994). It is known that JA signaling antagonizes SA signaling, a major component of the resistance against *P. syringae* (Kunkel and Brooks, 2002). COR is believed to take advantage of this antagonism to repress SA-mediated plant defense mechanisms. The mode of action of COR is described in more detail in the next section.

Many plant gram-negative pathogens are also able to directly inject virulence proteins, known as type III effectors (T3Es), directly into the plant cell through their type

III secretion system (TTSS) (Block et al., 2008). In general, plant pathogenic bacteria defective in TTSS are unable to successfully infect their host, demonstrating the importance of the TTSS for pathogenicity. T3Es promote disease in a number of ways. T3Es have been shown to suppress plant defense, trigger water and nutrients release from plant cells, or facilitate T3Es secretion into the cells. However, the function and target of only a fraction of the known T3Es have been identified. This work is complicated by the fact that some T3E have multiple targets and can interfere with plant defense mechanisms at different level. For example, the kinase inhibitor AvrPto from *P. syringae pv. tomato* has been shown to suppress PTI by binding to the Flg22 receptor FLS2 and inhibiting its autophosphorylation, which is necessary for activation of the downstream MAPKs (Xiang et al., 2008). AvrPto is recognized by the tomato R-protein Pto. This interaction is sensed by another R-protein, Prf, which triggers ETI (Salmeron et al., 1996). In addition to its activity on FLS2, AvrPto has also been shown to inhibit Pto kinase activity. However, this activity has no effect on Pto-Prf mediated ETI (Xing et al., 2007). Another example of the complex mode of action of T3Es is the ubiquitin ligase from *P. syringae*, AvrPtoB. AvrPtoB has been shown to suppress PTI by directly targeting FLS2 for degradation by the proteasome (Gohre et al., 2008). Prf, as in the case of AvrPto, also detects the interaction of AvrPtoB with Pto to trigger ETI (Mucyn et al., 2006). Truncated AvrPtoB proteins lacking the E3 ligase domain also trigger ETI through its recognition by the tomato protein kinase Fen. This recognition is also sensed by Prf to trigger ETI. However, AvrPtoB has been shown to ubiquitinate the Fen protein, precisely via its E3 ligase domain. This ubiquitination leads to the degradation of Fen and blocks the Prf-mediated ETI (Rosebrock et al., 2007). Those data suggest that AvrPtoB evolved

its E3 ligase domain to avoid the Prf-mediated ETI. Unlike Fen, a recent report shows that Pto avoids the AvrPtoB-mediated ubiquitination by phosphorylating and inactivating AvrPtoB within its E3 ligase domain providing a new mechanism by which plants defend themselves (Ntoukakis, in press).

As mentioned previously, the cell wall is an essential part of PID. Many pathogens, especially in the process of extracting water and nutrients from necrotic tissues, are known to secrete a battery of cell-wall degrading enzymes (CWDEs). Examples of CWDEs are cellulases, pectinases and proteases. The importance of these CWDEs for pathogenicity is well established for soft-rot pathogens such as *Erwinia carotovora* (Whitehead et al., 2002). The *P. syringae* genome also encodes several putative CWDEs. However, the role of these proteins during pathogenesis is not known. It is likely that CWDEs are not as important for *P. syringae* pathogenicity since this bacterium does not rely on macerated tissue to extract nutrients. It is possible that in the case of *P. syringae*, CWDEs play a role during its saprophytic phase in the soil, to degrade cell wall components from dead plants.

Finally, many pathogens have been shown to manipulate plant hormone signaling pathways to deregulate defense mechanisms and to promote symptoms. For example, a lot of pathogens are known to produce, or to induce the production by the plant, of auxin, a major developmental hormone (Spaepen et al., 2007). The tumorigenic bacteria *Agrobacterium tumefaciens*, responsible to the grown gall disease, is a good example. The tumors induced by *A. tumefaciens* partly result from the over-production of auxin induced by auxin biosynthetic genes coded by the *A. tumefaciens* T-DNA plasmid, and from the increased sensitivity of the host to auxin (Akiyoshi et al., 1983; Deeken et al.,

2006). In addition, *A. tumefaciens* was shown to suppress the HR response via the production of auxin (Robinette and Matthysse, 1990). In the case of non-tumorigenic bacteria, such as *P. syringae*, auxin is emerging as an important player in virulence. First, auxin treatment increases the susceptibility to *P. syringae* and the *P. syringae* genome codes for several genes involved in auxin metabolism (Chen et al., 2007; Glickmann et al., 1998; Yamada, 1985). Second, free auxin levels increase during *P. syringae* infection (Chen et al., 2007). In addition, Flg22 has been shown to repress auxin signaling via a miRNA mechanism suppressed by *P. syringae* (Navarro et al., 2006). However, the exact role of auxin signaling in increasing susceptibility to *P. syringae* is poorly understood. Another example of pathogens modulating plant hormone physiology is the production of coronatine (COR) by *P. syringae*. COR is the most investigated phytotoxin and is the subject of the next section.

IV. Mode of action of Coronatine (COR)

The phytotoxin COR was first isolated from a fermentation broth of the phytopathogenic bacterium *P. syringae atropurpurea* in 1977 (Ichihara et al., 1977). It is a polyketide produced by *P. syringae* that is made of two components, coronafacic acid (CFA) and coronamic acid (CMA), linked by an amide bond. CFA is an analog of jasmonate and CMA is structurally close to 1-aminocyclopropane-1-carboxylic acid (ACC), the precursor to ET, and to the amino acid isoleucine (Ile) (Bender et al., 1999). COR plays an important role in *P. syringae* virulence. Indeed, coronatine deficient mutants of *P. syringae* grow to lower titers in plant tissues and elicit less severe disease symptoms (water soaking lesions and chlorosis) than the wild-type in various plants

including *Arabidopsis* and tomato (Brooks et al., 2004; Mittal and Davis, 1995). Mutants in either CFA or CMA biosynthesis show reduced growth *in planta* and reduced symptoms showing that both are necessary for COR-mediated virulence.

COR is believed to be responsible for the chlorotic halo surrounding the necrotic lesions of speck diseases induced by *P. syringae* (Bender et al., 1999). The mechanism explaining chlorosis formation by COR is not fully understood, but COR was shown to repress photosynthetic genes in tomato and activate the expression of a chlorophyllase in *Arabidopsis* involved in chlorophyll degradation (Benedetti and Arruda, 2002). Recently, COR was shown to activate the production of ROS in a light-dependent manner that could also contribute to chlorotic lesion formation (Uppalapati et al., 2007). The role, if any, of COR-induced chlorosis in virulence is not known. On one hand, cell death occurring during the formation of chlorosis could provide the pathogen with nutrients. On the other hand, the chlorosis, reminiscent of the HR, could well be a host defense mechanism to contain COR-producing pathogens. In addition to chlorosis, COR has been shown to induce a cell hypertrophy, induce production of volatile secondary metabolites, inhibit root growth, trigger the production of ET and the accumulation of protease inhibitors (Bender et al., 1999). It is still not known if all those responses are direct or indirect effects of COR and their possible functions remains largely unknown.

As mentioned before, COR acts as a mimic of JA. Like JA, COR binds to the E3 ubiquitin ligase COI1 (Figure 2) (Katsir et al., 2008). COI1 is a major component of the JA signaling pathway and *coi1* mutants are severely impaired in multiple JA responses. It is interesting to note that *coi1* stands for *coronatine insensitive 1* and was first isolated in a screen for *Arabidopsis* mutants showing reduced growth inhibition by COR (Feys et

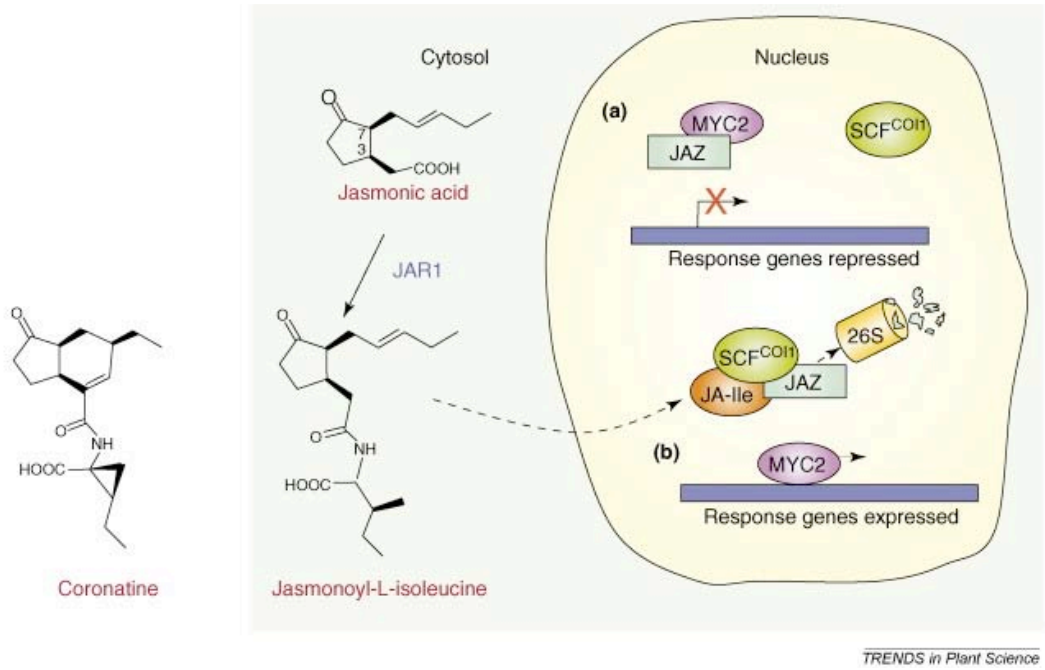

Figure 2. JA and COR signaling in *Arabidopsis.*
(a) MYC2 is blocked from activating expression of JA-regulated genes by the interaction of JAZ proteins. **(b)** Jasmonic acid is conjugated with isoleucine by JAR1 to form jasmonoyl-isoleucine (JA-Ile). JA-Ile (or COR) binds to COI1 and promotes SCF^{COI1} interaction with JAZ proteins, triggering their ubiquitination and degradation by the 26S proteasome. MYC2 is thereby released from its repression by JAZ proteins and regulates the expression of genes involved in jasmonate responses. Figure from Staswick et al., 2007.

al., 1994). The binding of COR to COI1 promotes the interaction of JAZ proteins (for Jasmonate ZIM-domain) with the SCF^{COI1} ubiquitin ligase complex and their targeting to the proteasome (Figure 2) (Katsir et al., 2008). JAZ proteins are known to be repressors of the transcription factor JIN1/MYC2 (for Jasmonate INsensitive 1) (Chini et al., 2007; Thines et al., 2007) and *MYC2* is an important transcription factor involved in many jasmonate responses (Lorenzo et al., 2004).

It is well established that JA and SA signaling are mutually antagonistic. Indeed, JA treatment or a stress that induces JA signaling (such as wounding or infection by a necrotrophic pathogen) suppresses the activation of SA-responsive genes such as *PR1*. Conversely, SA treatment or a stress that induces SA signaling (such as infection with a biotrophic pathogen) suppresses the activation of JA-responsive genes such as *PDF1.2* (Koornneef et al., 2008; Koornneef and Pieterse, 2008). Accordingly, *coi1* and *jin1* mutants, impaired in JA signaling, show an increase in SA-signaling and therefore are more resistant to *P. syringae*, which strongly activates SA signaling pathways (Kloek et al., 2001; Laurie-Berry et al., 2006). *P. syringae* is thought to exploit JA-SA antagonism by synthesizing COR to suppress SA-signaling and thereby increase pathogen virulence. Indeed, COR treatment activates JA signaling and suppresses SA responses (Kunkel and Brooks, 2002; Laurie-Berry et al., 2006). Moreover, it was shown that the increased resistance of *coi1* and *jin1* mutants is due to SA-signaling since the introduction of *nahG* transgene (a *P. Putida* gene encoding salicylate hydroxylase that converts SA into catechol) or a mutation in SID2 (which encodes the SA biosynthetic gene isochorismate synthase), restore normal growth of *P. syringae* in *coi1* or *jin1* mutants (Kloek et al., 2001; Laurie-Berry et al., 2006). Finally, SA levels are higher after infection with COR

deficient mutants compared to wild-type in tomato plants (Uppalapati et al., 2007), showing that COR may suppress SA production. Interestingly, *coi1nahG* or *jin1sid2* plants do not develop typical disease symptoms (in particular chlorosis) during *P. syringae* infection (Kloek et al., 2001; Laurie-Berry et al., 2006). In addition, in *sid2* and *nahG* plants, coronatine deficient mutants of *P. syringae* are still impaired for lesion formation (Block, 2005). Those data indicate that COR could promote lesion formation independently of SA-signaling through an unknown mechanism.

Another example of JA-SA antagonism is the phenomenon known as systemic acquired sensitivity or SIS. *P. syringae* was shown to induce systemic susceptibility to secondary *P. syringae* infection in *Arabidopsis*. SIS is caused by the production of coronatine (COR) and is a consequence of the mutually antagonistic interaction between JA and SA signaling pathways (Cui et al., 2005).

In natural conditions, endophytic pathogens such as *P. syringae*, enter the mesophyll layer through the stomata. Upon MAMP recognition, *Arabidopsis* closes its stomata, preventing bacteria from entering into the stomatal chamber and the mesophyll layer of the leaf (Melotto et al., 2006). MAMP-elicited stomatal closure is dependent on SA signaling since *nahG* and *sid2* plants are impaired in this defense response. In addition, SA itself triggers stomatal closure. Interestingly, *P. syringae* bacteria are able to evade this PTI response via the production of COR (Melotto et al., 2006). MAMP-induced stomatal closure is a good example of an SA-dependent mechanism blocked by COR.

V. Plant-microbe interactions in roots

V.1. Root Pathogens

Roots have received much less attention than leaves with respect to plant defense mechanisms against bacterial pathogens. This is due to a variety of factors. One is historical. Disease symptoms on the aerial part of the plants are more visible and therefore easier to describe, probably explaining why they received attention earlier. A second reason is technical. Plants are generally grown in soil, making the roots difficult to observe, isolate and wash without damage. The development of various hydroponic systems has allowed scientists to circumvent this problem. However, hydroponic systems do not allow the study of roots in their natural habitat. A third reason is that many microbes from the rhizosphere, the biologically active zone of soil surrounding the roots, cannot be cultured in the laboratory. Finally, compared to leaves, very few bacteria have been shown to successfully infect roots. *Ralstonia solanacearum*, which causes a bacterial wilt in a wide range of hosts, and *Agrobacterium tumefaciens,* responsible for the crown gall disease and the hairy root disease, are a couple of examples root bacterial pathogens. However, *Xanthomonas*, *Erwinia,* and most importantly *Pseudomonas* pathovars, the most studied plant pathogens, are leaf pathogens and are generally not considered to be root pathogens. This presents a practical experimental problem because many genetic and genomic tools have been developed for well-studied leaf pathogens but not root pathogens.

Although pseudomonads are not generally described as root pathogens, they have been shown to actively colonize roots. Therefore, their lack of pathogenicity in roots is

not due to physical proximity. Successful root pathogens are, for the vast majority, fungal pathogens belonging to genera such as *Phytophtora*, *Pythium* and *Fusarium* or filamentous bacteria such as *Streptomyces* (Okubara and Paulitz, 2005). Most of the work related to plant defense mechanisms in roots has been done using those pathogens. Most fungal root pathogens are necrotrophs, killing the roots using toxins and CWDEs like the oomycete *Pythium* and the basidiomycete *Rhizoctonia.* These pathogens feed on dead plant tissue (Okubara and Paulitz, 2005). However, some are hemibiotrophs and form haustoria to extract nutrients from living cells. This is the case of *Phytophtora sojae*. In general fungal root pathogens can infect a wide range of plant species (Okubara and Paulitz, 2005). Pathogenic *Streptomyces* species are believed to infect roots through short specialized infection hyphae (Loria et al., 2003). This penetration is facilitated by the production of thaxtomin, a toxin inhibiting cellulose deposition (Loria et al., 2003). *Ralstonia solanacearum* is a Gram-negative bacterium causing a wilt disease in diverse plants (Hayward, 1991). *R. solanacearum* cells attach to the root epidermis, especially at the root elongation zone and at the junction between the main root and lateral roots (Vasse, 1995). They can penetrate in the intercellular spaces of the root cortex and reach the vasculature. The bacteria then travel to the leaves and infect the rest of the plant. The mechanisms allowing *R. solanacearum* to penetrate and proliferate in the roots and the xylem vessels involve the secretion of CWDEs, T3Es, lipopolysaccharides and chemotaxis (Denny, 1991; Poueymiro and Genin, 2009; Vasse, 1995; Yao and Allen, 2006).

V.2. Plant Growth Promoting Bacteria

Even though roots have received less attention than leaves for pathogenic microbial interactions, beneficial plant-microbes interactions have been extensively studied in roots compared to shoots. These beneficial microbes include mycorrhizal fungi, N_2-fixing bacteria (such as *rhizobium*), and other plant-growth promoting rhizobacteria (PGPR) from the *Pseudomonas* and *Bacillus* genera. Mycorrhizas are symbioses between plant roots and fungi. The fungi provide the plant with water and minerals and the plant supplies the fungi with nutrients, mainly carbohydrates. In addition, mycorrhizal fungi are believed to protect the roots against pathogens by producing antimicrobial compounds, inducing roots defense mechanisms, and competing with pathogens for ecological niches (Morgan et al., 2005).

The best-studied case of N_2-fixing bacteria is *Rhizobium*. This bacterium has been shown to form specialized structure, called nodules, on legume roots. The formation of these nodules requires the production of flavonoids by the plant, that induce the secretion of lipo-oligosaccharides called Nod factors (Morgan et al., 2005). Nod factors are recognized by plant receptors and induce cell multiplication in the root cortex leading to nodule formation. Nod factors also induce the encapsulation of *Rhizobium* by root hairs. *Rhizobium* is subsequently able to enter cortical cell through a specially synthesized conduit called an infection thread. Once inside root cortical cells, the bacteria differentiate into bacteroids and fix atmospheric N_2 into NH_4^+, which is made available to the plant. *Pseudomonas* and *Bacillus* PGPRs are another example of beneficial root microbes. These bacteria colonize the surface of roots and are able to

control plant diseases. Therefore, PGPRs are considered biocontrol agents. Niche exclusion, production of antimicrobials, induction of plant defense, and competition for nutrients are believed to be mechanisms by which PGPRs exclude potential pathogens (Whipps, 2001). In particular, the production of siderophores by some PGPRs was shown to suppress soilborne diseases by competing for iron (Whipps, 2001). In addition to those local responses, some PGPRs have been shown to induce a systemic resistance from root to shoot, a mechanism referred as induced systemic resistance (ISR) (Pieterse et al., 1998; van Loon et al., 1998). ISR is described below in more detail.

VI. The plant defense mechanisms in roots

Not much is known about plant defense mechanisms in roots compared to shoot. JA and ET signaling are known to be important players against root necrotrophic pathogen. Indeed, the triple mutant *fad3fad7fad8*, unable to produce JA, the *jar1* mutant, impaired for JA signaling, as well as the *etr1* and *ein2* mutants, insensitive to ET, were all shown to be more susceptible to various *Phytium* pathovars (Geraats et al., 2002; Staswick et al., 1998; Vijayan et al., 1998). *R*-gene mediated resistance has been described for a few root-pathogen interactions. For example, the NBS-LRR tomato *I-2* gene has been shown to confer resistance against *Fusarium oxysporum* (Ori et al., 1997; Segal et al., 1992). However, the typical HR seen in leaves has not been described in roots. It is possible that some HR components are missing in the root or that plants actively suppress HR in the roots for unknown reason. This correlates with the lack of strong evidence for SA-mediated local defense responses in roots against pathogens. This is not due to the lack of SA-signaling in the roots since SA was recently shown to

play a role in resistance against root-knob nematodes (Wubben et al., 2008). In addition, published microarray data have shown that SA treatment in roots activates a number of genes involved in plant defense (Badri et al., 2008), a result confirmed by microarrays performed in Frederick Ausubel laboratory by graduate student Wisuwat Songnuan (unpublished). Indeed Wisuwat has shown that SA induces more genes in *Arabidopsis* roots than in the shoots.

As mentioned before, root colonization by some PGPRs can trigger a systemic resistance response from root to shoot against a broad spectrum of fungal and bacterial pathogens in addition to some herbivorous insects and viruses. This mechanism, known as induced systemic resistance (ISR), is mediated by JA and ET signaling, but also requires the transcriptional regulator NPR1 and the transcription factor MYB72 in *Arabidopsis* (Pieterse et al., 1998; Van der Ent et al., 2008). Transcriptional analysis has shown that root colonization by PGPRs does not trigger strong transcriptional changes in the leaves but instead primes the tissues for a stronger defense response in case of pathogen attack (Conrath et al., 2006). This priming is believed to limit energy costs for the plant. The mechanisms underlying the elicitation of ISR are still unclear, but increasing evidence suggests that MAMPs are involved in that process (Bakker et al., 2007). Indeed, flagella from *P. putida* WCS358 was shown to trigger ISR against *P. syringae* in *Arabidopsis* (Meziane, 2005). However, a *P. putida* mutant lacking flagella was still able to trigger ISR, showing that other MAMPs are also able to induce this response. It is likely that LPS from *P. fluorescens* WCS417r also induces ISR against *P. syringae* in *Arabidopsis* (Van Wees et al., 1997). In addition to MAMPs, siderophores, salicylic acid and the antibiotic 2,4-diacetylphloroglucinol (DAPG) produced by some

Table 2. Molecular determinants of ISR*

Molecular Determinant	bacterial strain	Host plant	References
Flagellin	*P. putida* WCS358	*Arabidopsis*	(Meziane, 2005)
Lipopolysaccharides	*P. fluorescens* WCS374	Radish	(Leeman, 1995)
	P. fluorescens WCS417	*Arabidopsis*, radish	(Leeman, 1995; Van Wees et al., 1997)
	P. putida WCS358	*Arabidopsis*, bean, tomato	(Meziane, 2005)
Lipopeptides surfactin and fengycin	*B. subtilis* Bs168	Bean, tomato	(Ongena et al., 2007)
2,3-butanediol	*B. subtilis* GB03	*Arabidopsis*	(Ryu et al., 2004)
N-alkylated Benzylamine	*P. putida* BTP1	Bean	(Ongena et al., 2005)
Pseudobactin siderophore	*P. fluorescens* CHA0	Tobacco	(Maurhofer, 1994)
	P. fluorescens WC374	Radish	(Leeman, 1996)
	P. putida WCS358	*Arabidopsis*, bean, tomato	(Meziane, 2005)
Pyocyanin, pyochelin, salicylic acid	*P. aeruginosa* 7NSK2	Tomato	(Audenaert et al., 2002)
2,4-diacetylphloroglucinol (2,4-DAPG)	*P. fluorescens* CHA0	*Arabidopsis*, tomato	(Iavicoli et al., 2003)

*Adapted from Bakker et al., 2007.

PGPRs have been shown to be involved in ISR (Bakker et al., 2007). Table 2 summarizes some molecular determinants from PGPRs that were shown to trigger ISR.

VII. This Thesis

Despite the fact that roots are the organs most subject to microbial interactions and that MAMPs seem to play an important role in root defense mechanisms as has been described for ISR in the previous section, MAMP signaling in roots has received much less attention than in the leaves. One possible explanation is that MAMP signaling was first studied for endophytic bacterial pathogens like *P. syringae* and that the MAMP signaling research "field", being relatively new, has not yet had time to explore the roots. Major questions need to be answered regarding MAMP signaling in roots:

- Are the MAMP signaling components conserved between roots and shoots?
- Are MAMPs equally recognized in roots and shoots?
- Are the MAMP-elicited defense mechanisms that are triggered in shoots also triggered in roots? For example, FLS2 is expressed in roots, but callose deposition after Flg22 elicitation has been reported in leaves, stems and cotyledons but not in roots. Part of my thesis was to investigate in detail the response to various MAMPs in the roots of *Arabidopsis* to try to answer those questions.

Another major aspect of my thesis research was to investigate whether bacterial pathogens are able to suppress the MAMP response in the roots as they do in leaves. If this is the case, it would be interesting to know if they do so using T3Es, similar to what takes place in the leaves, or via other mechanisms. PGPRs, like any microbes, produce MAMPs and it is possible that they evolved strategies, similar to pathogens, to evade PTI

in order to successfully colonize the roots.

The first chapter of this thesis focuses on the development of a fast, high throughput seedling assay to study plant-microbe interactions in *Arabidopsis* seedlings. This assay, developed in collaboration with my fellow graduate student Wisuwat Songnuan, provided us with an alternative model that combines many of the advantages of the more traditional assays used to study MAMP signaling and pathogen infection in protoplasts and adult plants. It also enabled me to extensively study the MAMP response in roots, the main subject of this thesis.

The second chapter of my thesis concentrates on the MAMP response in *Arabidopsis* roots and its suppression by *P. syringae* via the production of COR. In particular, my work focused on callose deposition, a well-known innate immune response triggered by MAMPs during PTI. I found that the MAMPs Flg22, PGN and chitin activate the expression of several GUS reporter genes and the deposition of callose in specific parts of the roots. Flg22 and PGN induce a response in the elongation zone (EZ) of the roots, whereas chitin activates MAMP signaling in the mature part of the roots. I provide evidence that this response is independent of SA signaling, but requires the transcription factor MYB51, the cytochrome P450 CYP81F2, and the myrosinase PEN2. In addition, I show that ET signaling significantly potentiates the Flg22 response in the roots. Finally, I demonstrate that *P. syringae* suppresses the MAMP response in the roots using COR via the E3 ubiquitin ligase COI1 and the transcription factor MYC2. This property of COR allowed me to perform a forward genetic screen to isolate new mutants impaired for the COR-mediated suppression of the MAMP response in roots.

Finally, the third chapter of this thesis focuses on *CYP76C2*, a cytochrome P450

highly induced by MAMPs that is involved in the plant response to cell death mechanisms (Godiard et al., 1998). This chapter describes interesting characteristics regarding *CYP76C2* expression and regulation and provides hints regarding its function. I confirmed that *CYP76C2* is induced in all tissues undergoing active cell death and I found that this gene is regulated by SA-signaling. In addition, I provide evidence that this gene is involved in resistance against oxidative stress.

Chapter 2

A seedling assay for MAMP signaling and infection studies

A seedling assay for MAMP signaling and infection studies

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Most studies of the plant response to either pathogens or microbe-associated molecular patterns (MAMPs) have been carried out using either mature plants or plant tissue culture cells. To provide an alternative system to facilitate the study of defense signaling pathways, we have developed an Arabidopsis model that utilizes ten-day old Arabidopsis seedlings treated with MAMPs including oligogalacturonides (OGs) (Jin and West 1984; Aziz, Heyraud et al. 2004) or the synthetic flagellin peptide Flg22 (Felix, Duran et al. 1999) or with pathogens in multi-well plates. Using this system we have carried out transcriptional profiling studies, MAMP-elicited protection assays, MAMP pathway studies using reporters of the plant defense response, and metabolic profiling of root exudates.

Traditionally, plant-pathogen interactions are investigated by directly infecting mature plants using a variety of inoculation methods including leaf infiltration, dipping, or spraying. Symptoms are observed for several days after inoculation, usually accompanied by direct quantification of colony forming units (cfu), spores, or disease symptoms. Alternatively, different staining techniques are used to visualize disease progression and defense responses. While these approaches are similar in some respects to natural infection and have provided the vast majority of data in the field, they are time-consuming and labor-intensive. Growing mature plants usually takes a considerable amount of time and space. Moreover, it is difficult to maintain sterility when growing mature plants, allowing other microorganisms to potentially complicate the interpretation of experiments.

As an alternative to using mature plants, mesophyll protoplasts can be isolated from mature plants and transfected with desired transient expression

constructs to observe rapid changes in the defense responses at the molecular and cellular levels (He, Shan et al. 2007; Yoo, Cho et al. 2007). Protoplast assays have provided insights into many processes that are difficult to investigate in whole plants. They facilitate the study of a particular gene construct when a stable transformant is not yet available and they are cost-effective and semi-sterile. However, they require extensive training and are very sensitive to the particular conditions under which the plants that provide the source of protoplasts are grown. Moreover, it is difficult to obtain healthy protoplasts from cells other than mesophyll cells, and it is not readily feasible to use protoplasts for organ-specific or systemic signaling studies. Finally, protoplasts have relatively short lives after harvest, and are not suitable for experiments that require a long time course.

We have developed an Arabidopsis seedling assay to study MAMP signaling that combines many of the advantages of using mature plants or protoplasts. We have shown that ten-day old seedlings can be used for experiments instead of four-week or older plants that are needed either for harvesting protoplasts or for traditional infection methods. The seedlings are germinated and grown in liquid medium under sterile conditions. In a typical seedling assay, each well of a 12-well plate contains 10-15 seedlings, providing enough replicates to average out biological variations. Many different experimental treatments can be carried out in a single tissue culture plate. For high-throughput assays, seedlings can be grown in 96-well plates and used in genetic or chemical screens. Chemicals, hormones, elicitors, or pathogens can be added directly into the medium. If staining is required, it can be done conveniently in the wells where seedlings are grown. Whole seedlings can be used to study signaling between cells or defense responses in specific tissues. Below, we describe a series of experiments in which we have validated the seedling system by demonstrating that seedlings respond in a similar manner as mature plants in a variety of pathogenicity-related assays.

We typically distribute 10-15 vernalized sterile seeds into each well of a 12-well tissue culture plate containing 1 ml of filter-sterilized Murashige and Skoog Basal medium supplemented with 0.5% sucrose, pH 5.8. The plates are wrapped with parafilm to prevent evaporation and placed at 22^{o}C under a 16 hours light/ 8 hours dark photoperiod with a light intensity of 100 $\mu Em^{-2}s^{-1}$. After 8 days, the medium is replaced with a fresh batch to replenish the nutrients and equalize the volume of liquid in the wells. On day 10, seedlings can be treated by adding desired concentration of hormones, MAMPs, or bacteria directly into the liquid medium. For *Psudomonas syringae* infection, bacteria are harvested in log phase, thoroughly rinsed with the plant medium,

resuspended to OD_{600} = 0.2, and10µl of the suspension is added to each well. The concentration of the starting inoculum can be adjusted as needed. We have noticed that *P. syringae* infection progresses more uniformly and faster if the plates are slowly shaken (30-50 rpm). Condensation on the lids of the assay plates is avoided to reduce the appearance of water-soaked lesions and to prevent contamination. For high throughput assays, 1-7 seedlings can be grown in each well of a 96-well plate containing 100 µL of medium. Figure 1 illustrates 12-well and 96-well seedling assays at day 10.

MAMP-mediated defense responses

We confirmed the validity of the seedling assay by analyzing MAMP-mediated induction of a variety of known MAMP-regulated genes, including *PR1*, *FRK1,* and *WRKY29* (Asai, Tena et al. 2002). Figure 2a shows that *WRKY29* is rapidly induced in seedlings treated with Flg22, corresponding to results obtained in mature plants or protoplasts. Similar results are obtained with many other MAMP-induced genes and with other MAMPs including OGs and the synthetic polypeptide elf18 that corresponds to a highly conserved region of bacterial elongation factor EF-Tu (Kunze, Zipfel et al. 2004). We have also carried out transcriptional profiling studies of MAMP-treated seedlings as described in another chapter in this volume (see Denoux *et al.* "Characterization of Arabidopsis MAMP response pathways")*.*

Our laboratory has also utilized the seedling assay to study MAMP-mediated induction of callose deposition in seedling cotyledons. As shown in Figure 3b, callose deposition induced by 1µM of Flg22 is readily detectable above the background level (Figure 3a) after staining with aniline blue(Gomez-Gomez, Felix et al. 1999). No callose deposition was observed when a Flg22 receptor mutant (*fls2*) (Gomez-Gomez, Felix et al. 1999) (Figure 3c) or a callose synthase mutant (*pmr4*) (Nishimura, Stein et al. 2003) (Figure 3d) were treated with Flg22. Because the seedlings are in contact

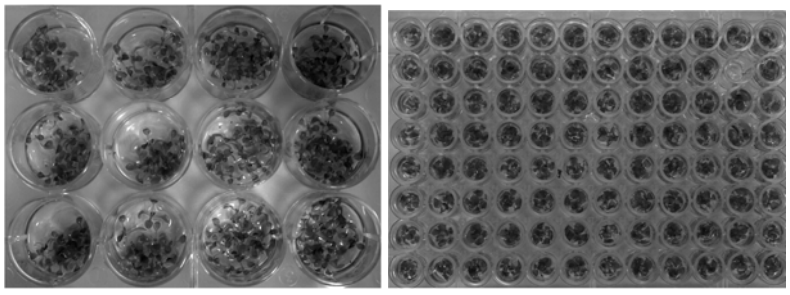

Fig. 1. 10-day-old seedlings in 12-well (a) and 96-well (b) formats.

with liquid media, they are more uniformly in contact with Flg22 and the stain, resulting in a more homogenous staining than other methods, such as leaf-infiltration. Moreover, the small-size of the cotyledons allow them to be viewed in one field under a microscope, preventing errors caused by selecting fields that are not representative of the entire leaves.

The seedling assay also has a clear advantage over mature plants and protoplasts for the study of genes that are expressed in roots. Root tissue can be isolated from seedlings and used for regular RT-PCR, quantitative RT-PCR, or Northern blots. Moreover, seedling roots can be readily stained if GUS or GFP reporter lines are available for particular MAMP-induced genes

Seedling assay for infection studies

As mentioned above, bacterial inoculation is simply carried out by adding the suspended bacterial cells directly into the media. Symptoms can be monitored for several days after inoculation. As seen in assays in mature plants, seedlings infected with virulent or avirulent *P. syringae* pv. *tomato (Pst)* strain DC3000 exhibit necrotic symptoms and die faster than those infected with the nonhost pathogen *P. syringae* pv. *phaseolicola* (*Psp)*

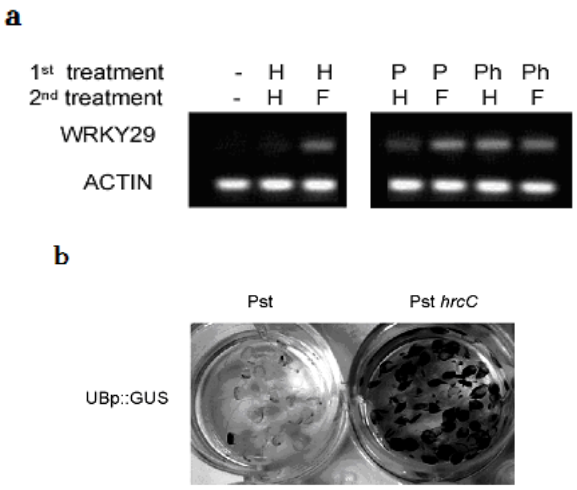

Fig. 2. Using the seedling assay to study MAMP-mediated *WRKY29* gene induction and suppression by the type III secretion system of DC3000 (a). Seedlings were inoculated with bacteria or H_2O for 18 hours followed by elicitation with 1μL Flg22 or H_2O for 1 hour before tissue collection and RT-PCR. (- = no treatment, H = H_2O, P = *Pst* DC3000, Ph = *Pst* DC3000 *hrpL* mutant, F = Flg22) (c) Suppression of *UB::GUS* by wild-type *Pst* DC3000 compared to *Pst* DC3000 *hrcC*, a type III secretion mutant.

NPS3121, or a *Pst* DC3000 *hrcC* mutant lacking the type III secretion apparatus (Figure 4a). Nonetheless, even the nonhost bacteria can stunt the growth of the seedlings significantly compared to the mock-treated seedlings.

The number of bacteria inside the seedlings can also be quantified in the seedling infection assays. Seedlings are washed briefly with 70% ethanol followed by sterile water and blotted dry on absorbent material before grinding in a 1.5mL eppendorf tube and plating for colony forming units. *Pst* DC3000 grows rapidly until about 24 hours after inoculation (Figure 4b). Interestingly, however, although the *hrcC* mutant appears to be less virulent than the wild-type in this assay, it is still able to grow relatively well inside the plants. Colony counts show that the *hrcC* mutant grows only about 10 fold less than the wild-type in the seedlings, whereas a *hrcC* almost entirely fails to grow in a mature leaf infiltration assay.

Using a GFP-marked *Pst* DC3000 strain, bacteria could be visualized inside the stomata and propagating in the apoplastic area. After 48 hours, bacteria colonized the intercellular spaces of the leaves and invaded the vascular system. Interestingly, it appears that *Pst* DC3000 is unable to penetrate the roots, but rather accumulates on irregular surfaces, such as areas close to root hairs or lateral roots. (Figure 4c).

Interestingly, none of these bacterial strains can grow in the MS liquid medium if the seedlings are not present, even though the medium contains plentiful nitrogen and sugar. An unidentified, but active interaction between

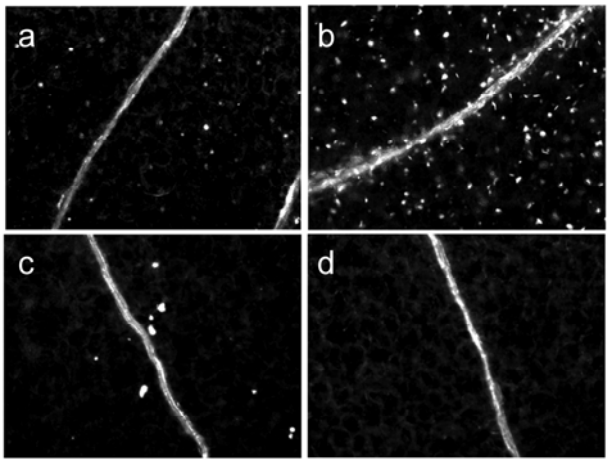

Fig. 3. Callose deposition elicited by Flg22 can be detected in seedling cotyledons (a) wild-type Col-0 treated with water, (b) wild-type Col-0 treated with 1 µM Flg22, (c) *fls2* mutant treated with 1 µM Flg22 (d) *pmr4* mutant treated with 1 µM Flg22. Seedlings were stained with 0.01% aniline blue in 0.15M K_2HPO_4, pH 9.5.

plants and bacteria must be occurring because the bacteria also fail to grow in media in which plants have been grown and they stop growing immediately if the seedlings are removed from the well. In the presence of the plants, the growth inside the plant is usually correlated with the growth in the media.

As previously shown by Zipfel *et al*. (Zipfel, Robatzek et al. 2004), Flg22 protects plants from subsequent infection by DC3000. We obtained a similar result in the seedling assay (Figure 5). In addition, we found that the level of protection increases as the time of pretreatment with Flg22 increases from 3 hours to 12 hours before inoculation. Interestingly, seedlings are better protected by pretreatment for 12 hours than 24 hours. We used the same

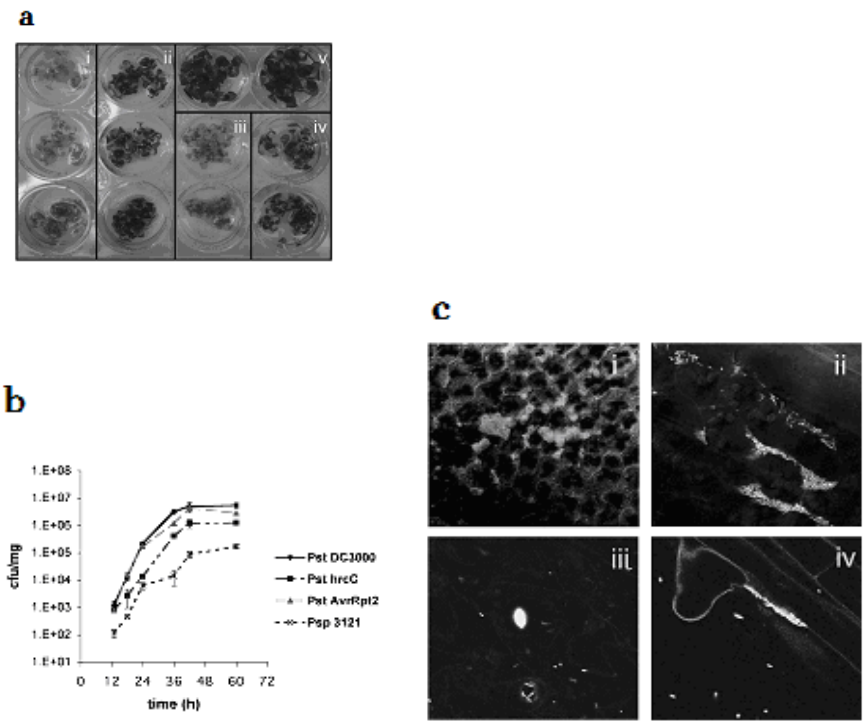

Fig. 4. Seedlings infected with Pseudomonas syringae. (a) Seedlings exhibit different degrees of necrotic symptoms after 5 days of infection with wild-type P. syringae pv. tomato strain DC3000 (i), a type III secretion mutant Pst DC3000 hrcC (ii), Pst DC3000 carrying the avirulence gene avrRpt2 (iii), a nonhost pathogen P. syringae pv. phaseolicola strain NPS3121 (iv), compared to mock-treated samples (v); (b) cfu quantification of bacteria inside the seedlings; (c) Confocal microscopy of seedlings infected with wild-type DC3000 carrying a constitutive GFP marker for 48 hours, showing bacteria proliferating in the apoplastic space (i) and (ii) and stomata (iii), but only accumulating on the surface of roots (iv).

approach to show that seedlings pretreated with OGs were also significantly protected from DC3000 infection.

It is known that pathogens such as DC3000 can suppress certain basal defense responses via transfer of protein effectors into host cells via the type III secretion system (Kim, da Cunha et al. 2005; de Torres, Mansfield et al. 2006). We could also observe this phenomenon in the seedling assay, using RT-PCR (Fig 2a) or GUS staining of reporter lines (Fig 2b). As shown in Figure 2a, *WRKY29* induction by a *Pst* DC3000 *hrpL* mutant is higher than by wild-type (lanes 4 and 6), even though both strains presumably synthesize the same MAMPs. Our interpretation of this result is that the *hrpL* does not suppress *WKRY29* because it fails to synthesize type III effectors. However, excess Flg22 overcomes DC3000-mediated suppression of *WRKY29* (lanes 5 and 7). Another example, shown with the GUS staining assay in Figure 2b, is the suppression of the *UB-GUS* reporter gene, identified by microarray analysis as strongly induced by Flg22 or OGs. This gene is induced highly by the *Pst* DC3000 *hrcC* mutant that lacks a structural component of a type III secretion needle, but the induction is hardly visible when treated with the wild-type strain of DC3000.

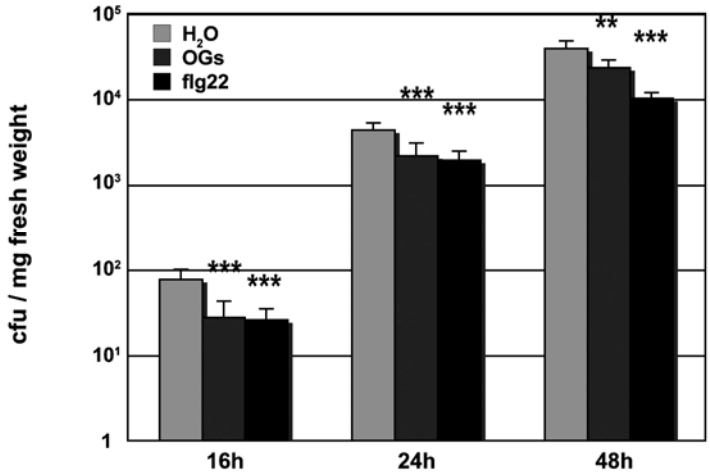

Fig. 5. Flg22 and OGs protect seedlings from infection by *Pst* DC3000. 10-day old seedlings were treated with H_2O, 200µM OGs, or 1µM Flg22 8 hours prior to inoculation with DC3000 at $OD_{600} = 0.0002$. Bacteria inside the seedlings were counted at times specified. (** = $P < 0.01$, *** = $P < 0.001$, *t*-test).

Limitations of the seedling assay

Despite many advantages, the seedling MAMP and infection assay has certain limitations. The seedlings are mostly submerged since germination and appear vitrified after several days. In the infection assay, entry of bacteria through stomata and growth in the intersticial spaces is presumably facilitated by the excess liquid around the seedlings. Also, unlike the leaf-infiltration assay, infecting bacteria might be able to leave and re-enter the plants multiple times. Finally, the seedlings can only survive for about two weeks in microtiter wells because of overcrowding and nutrient depletion.

Conclusions

We established and validated an Arabidopsis seedling assay for MAMP-mediated signaling and bacterial infection studies. The seedling assay provides an alternative model for experiments that require whole plants under sterile conditions. The assay can be carried out in a short amount of time, and/or a limited space. Seedlings can be used for several purposes, including RT-PCR, GUS staining of reporter lines, staining for callose deposition, observing symptoms after infection with different strains of bacteria or colony counting, and biological and chemical screens.

Acknowledgements

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Chapter 3

MAMP signaling in *Arabidopsis thaliana* roots and its suppression by the *Pseudomonas syringae* phytotoxin coronatine

I. Introduction:

Roots are surrounded by a biologically active zone extremely rich in microorganisms called the rhizosphere, making roots the plant organ most subject to microbial interactions. However, these root-microbe interactions are poorly characterized due to the facts that roots are relatively inaccessible and that many rhizosphere microbes are not cultivable in the laboratory. Despite these limitations, recent technical advances in high throughput sequencing of environmental DNA are giving new insights into microbial diversity in the soil. In addition, many diverse root-microbe interactions are now being studied. Root-microbe interactions can be beneficial, as in the case of Mycorrhizas or N_2-fixing bacteria, but they can also be pathogenic. Most root pathogens identified so far are fungi belonging to genera such as *Phytophtora*, *Pythium* and *Fusarium,* or filamentous bacteria from the *Streptomyces* genus (Okubara and Paulitz, 2005). Interestingly, few bacterial species have been shown to successfully infect roots, such as in the case of *Ralstonia Solanacearum*, which causes a bacterial wilt in a wide range of hosts (Hayward, 1991). *Agrobacterium tumefaciens* is another root-infecting bacterial species that is responsible for the crown gall and the hairy root diseases. Although many bacteria in the genus *Pseudomonas* are successful foliar pathogens, they have not been described as root pathogens, even though they are very successful root colonizers. Indeed, many *Pseudomonas* strains actually promote plant growth by protecting the roots against potential pathogens by competing for nutrients and ecological niches, producing antimicrobials, and inducing plant defense mechanisms (Whipps, 2001). In addition to the protective effect of plant growth promoting

Pseudomonas species in the roots, some of these bacteria trigger a systemic resistance against a broad spectrum of fungal and bacterial pathogens known as induced systemic resistance (ISR). ISR primes the activation of defense genes in leaves, allowing the plant to respond more strongly when attacked by a foliar pathogen (Pieterse et al., 1998; van Loon et al., 1998). ISR is mediated by jasmonate (JA) and ethylene (ET) signaling and requires the transcriptional regulator NPR1, a key regulator in salicylic acid (SA) signaling. Despite the fact that plants are constantly subject to a diverse range of microbial interactions in the roots, the molecular bases of these interactions are still unclear.

Plants have evolved an innate immune response to protect themselves against pathogens. Like animals, plants recognize conserved epitopes of microbe-derived molecules called microbe-associated-molecular patterns (MAMPs) such as bacterial flagellin (Felix et al., 1999) and bacterial elongation factor Tu (Kunze et al., 2004). Other MAMPs include chitin, a major component of the fungal cell wall, lipopolysaccharides (LPS) or peptidoglycans (PGNs). MAMP recognition is mediated by pattern recognition receptors (PRRs). In addition to an oxidative burst, ethylene and nitric oxide production, MAMP recognition triggers a complex cascade of MAP kinases that leads to the activation of many transcription factors and defense-response genes as well as the deposition of callose, a β(1-3)-glucan polymer, which strengthens and dams weak or compromised sections of plant cell walls at the site of pathogen attack. One of the best-characterized MAMPs is Flg22, a 22 amino acid synthetic polypeptide corresponding to a highly conserved epitope of the *Pseudomonas aeruginosa* flagellin protein (Felix et al., 1999). Flg22 is recognized by the leucine-rich repeat receptor like

kinase (LRR-RLK) FLS2 (Gomez-Gomez et al., 2001). Despite the facts that Flg22 is one of the best-studied MAMPs and that the roots are constantly subject to microbial interactions, very little is known about MAMP-mediated responses in roots.

Many pathogens have evolved strategies to evade the plant immune response, including, in the case of bacteria, the injection of virulence effectors directly into the plant cell using the type III secretion system (TTSS) (Block et al., 2008). Type III effectors play a key role in the virulence of pathogenic bacteria such as *Pseudomonas syringae* by suppressing the plant basal immune response that is activated by MAMP recognition. In addition, many *P. syringae* pathovars secrete the low molecular weight phytotoxin coronatine (COR) that functions as a mimic of JA-Isoleucine (JA-Ile), the active intracellular amino acid conjugate form of JA, taking advantage of a natural mutually antagonistic interaction between the SA and JA signaling pathways (Kunkel and Brooks, 2002). This results in the suppression of SA signaling, a key component in basal resistance against *P. syringae*. In addition, COR represses the Flg22-elicited activation of the *Arabidopsis* gene *NHO1*, which is important for resistance against non-host *Pseudomonas* strains (Li et al., 2005; Lu et al., 2001). By definition, non-host bacteria are *bone fide* pathogens on some hosts but not pathogenic on a particular host in question. Finally, COR suppresses MAMP-induced stomatal closure, believed to block epiphyte pathogens such as *P. syringae* from entering the interior of leaves through these natural openings (Melotto et al., 2006). This suppressive ability of COR to block SA signaling and stomatal closure is mediated by COI1, an E3 ligase involved in jasmonate signaling and a key component of the defense response against nectrophic pathogens and insect herbivores.

In this work, using GUS reporters corresponding to MAMP-activated genes and MAMP-elicited callose deposition responses, we show that three MAMPs, Flg22, chitin, and PGN, trigger a strong tissue-specific response in *Arabidopsis* roots. In particular, we show that the Flg22 and PGN responses are restricted to the elongation zone of the root tip, whereas the response to chitin is localized in the mature zone of the roots. We also demonstrate that the MAMP-triggered callose deposition in roots is dependent on indole glucosinolate biosynthesis, on the PEN2 myrosinase, and on ET signaling, similar to what was previously shown in cotyledons (Clay et al., 2009). In addition, we find that ET signaling plays a major role in the Flg22-elicited responses in roots by potentiating the root response to Flg22. Similar to what was described in leaves, we show that *P. syringae* suppresses MAMP responses in the roots, but unlike leaves, this suppression is not dependent on the TTSS but on the production of COR. Indeed, *cor*$^{-}$ mutants of *P. syringae*, unable to produce COR, do not suppress the Flg22 response whereas purified COR does. In contrast to the expectation that COR suppresses MAMP responses by antagonizing SA-activated defense pathways, we demonstrate that the MAMP-triggered callose deposition in roots is independent on SA signaling. Finally, the COR-mediated suppression of MAMP responses is dependent on COI1 and MYC2, two major players in the JA signaling pathway. This is the first reported example of a mechanism suppressed by COR in plant roots.

II. Materials and Methods

II.1. Plant growth conditions

To carry out either callose deposition or GUS reporter gene staining assays in the roots, *Arabidopsis thaliana* Col-0 seedlings were grown in 12-well microtiter dishes sealed with parafilm, each well containing 10 to 15 plants and 1mL Seedling Growth Medium (SGM; 1X MS basal medium with vitamins (Phytotechnology Laboratories) containing 0.5g/L MES hydrate, and 0.5% sucrose at pH5.7). Plants were grown for 10 days at 22°C in a plant growth chamber under 16 hours of light (100μE). The medium was changed on day 8.

For experiments involving root RNA extraction, in order to easily separate the roots from the shoots, plants were grown vertically in 20x100mm circular Petri dishes containing 25mL of SGM medium solidified with 1% phytagar (PlantMedia) for 2 weeks at 22°C in a plant growth chamber under 12 hours of daylight (100 μE). The plates were then placed horizontally and covered with 6mL of SGM medium for 2 days before treatment with elicitors and extraction.

II.2. Bacterial strains and infections

Pseudomonas syringae bacterial strains were cultured on KB plates supplemented with appropriate antibiotics: 50μg/mL rifampycin for *P. syringae* pv. *tomato (Pst)* DC3000 and *Pst* CUCPB5112 (*hrcC*), 50μg/mL kanamycin for *Pst*DB4G3 (*cfa6*), *Pst*DB29 (*cfa6cmaA*) and *P. syringae* pv. *maculicola (Psm)* ES4326-*cfa6*, 30μg/mL streptomycin for *Pst*AK7E2 (*cmaA*) and *Psm*ES4326. For infection of seedlings grown

in 12-well microtiter dishes, bacteria were grown overnight in KB supplemented with an appropriate antibiotic at 28°C. Bacteria were centrifuged, washed three times with water, and resuspended in water to a final OD_{600} of 0.04. 10 day-old seedlings were infected by adding 50µL of bacterial suspension into each well to a final OD_{600} of 0.002. Treatments with elicitors were performed 18h or 6h after infection. The *Pseudomonas aeruginosa* PA14 and *E. coli* DH5α bacterial strains were cultured on LB plates and grown overnight at 37°C. Infection of seedlings by PA14 or DH5α was also performed with an initial OD_{600} of 0.002. *Pst* strains DB29, DB4G3 and AK7E2 carry a transposon expressing the *uidA* gene coding for the β-glucuronidase, which interferes with the $Arabidopsis_{pro}$*:GUS* reporter assays. For experiments involving these strains, 18h after inoculation, the media from each seedling assay was collected and filtered through a 0.22µM filter (Millipore). Fresh 10-day old seedlings were then treated with this "bacteria free" media and the various elicitors.

II.3. Treatment of seedlings with elicitors, hormones, toxins, and inhibitors

Elicitors, hormones, toxins, or inhibitors were used at the following concentrations unless otherwise specified: 100nM Flg22 for GUS assays; 1µM Flg22 for callose assays and root RNA extraction; 1µM Elf26; 100µg/ml *B. subtilis* peptidoglycan (Sigma); 100µg/mL chitin (Sigma) for GUS assays; 500µg/mL chitin for callose assays; 1µM coronatine (Sigma); 10µM methyl-jasmonate (Sigma); 1µM K252a (Sigma); 100µg/mL BFA (brefeldin A) (Sigma); 100µM SA; 100µM ACC (1-aminocyclopropane-1-carboxylic acid). A 10mg/mL chitin stock solution was prepared by autoclaving

250mg of chitin resuspended in 25mL of water for 30 minutes. The solution was then centrifuged and the supernatant collected.

II.4. Root RNA extraction and RT-PCR and qRT-PCR analysis

Total RNA was extracted from the roots of approximately 15 two week-old seedlings per sample using TRIzol (Invitrogen) according to the manufacturer's instructions. The roots were snap frozen by liquid nitrogen and grinded using a mortar and pestle. Total RNA was treated with DNAse I (Ambion) to avoid genomic DNA contamination and 1μg of total RNA was reverse-transcribed using the iScript cDNA synthesis kit from Biorad. qRT-PCR was performed using a CFX96 real-time PCR machine (Biorad) and iQ SYBR Green Supermix (Biorad). The program used for qRT-PCR was as follows: 3 minutes at 95°C, 45 cycles of 15 seconds at 95°C/30 seconds at 53°C, followed by a melt curve from 70°C to 94°C with 0.5°C increments every 10 seconds. Primers used for qRT-PCR were as follows:

CYP71A12-F, GATTATCACCTCGGTTCCT

CYP71A12-R,CCACTAATACTTCCCAGATTA

MYB51-F, ACAAATGGTCTGCTATAGCT

MYB51-R, CTTGTGTGTAACTGGATCAA

ERF1-F, TCGGCGATTCTCAATTTTTC

ERF1-R, ACAACCGGAGAACAACCATC

EIF4A1-F, TCTGCACCAGAAGGCACA

EIF4A1-R, TCATAGGATGTGAAGAACTC

II.5. GUS Histochemical assay

After treatment with bacteria and/or elicitors, etc., plants grown in 12-well microtiter dishes were washed with 50mM Sodium Phosphate buffer pH7. 1mL of GUS substrate solution (50mM Sodium Phosphate pH7, 10mM EDTA, 0.5mM $K_4[Fe(CN)_6]$, 0.5mM $K_3[Fe(CN)_6]$, 0.5mM X-Gluc, 0.01% Silwet L-77) was poured in each well. The plants were vacuum-infiltrated for 5min and then incubated at 37°C for 4 hours otherwise specified. Tissues were fixed with a 3:1 ethanol:acetic acid solution at 4°C overnight and placed in ethanol 95%. Tissues were cleared in lactic acid and observed under a Discovery V12 microscope (Zeiss).

II.6 Callose staining

Following treatment with elicitors, bacteria, etc., 10 day-old seedlings grown in 12-well microtiter dishes were fixed in a 3:1 ethanol:acetic acid solution for several hours. The seedlings were briefly vacuum-infiltrated and the fixative was changed several times to insure both thorough fixing and clearing of the tissues, which is essential for good callose detection in the roots. Seedlings were re-hydrated in 70% ethanol for 2 hours, 50% ethanol for an additional 2 hours, and water overnight. After 2 or 3 water washes, seedlings were treated with 10% NaOH, vacuum-infiltrated briefly and placed at 37°C for 1 to 2 hours in order to make the tissues transparent. This last step was also very important for callose detection. After 3 or 4 water washes, seedlings were incubated in 150mM K_2HPO_4, pH9.5, 0.01% aniline blue (sigma) for several hours. The roots were mounted on slides in 50% glycerol and callose was observed immediately after using an Imager Z.1 microscope (Zeiss) under UV (excitation 390nm, emission 460nm).

II.7. Construction of Transgenic Lines

1.7 to 2.5 kb of the promoter regions of *MYB51* (1.7 kb), *WRKY11* (1.7 kb), *AT5G25260* (2.5 kb), *CYP71A12* (2.5 kb) or *PEN2* (2 kb) were amplified using Expand High Fidelity polymerase (Roche) and cloned into the multiple cloning site of pBI101, which confers resistance to kanamycin. The *promoter:GUS* constructs were then sequenced and transformed into *Agrobacterium tumefaciens* strain GV3101. Columbia wild type plants were then transformed and progeny selected on kanamycin as described (Clough and Bent, 1998). The primers used to amplify the different promoters were as follows (the restriction site used is underlined and the enzyme indicated in parentheses):

p71A12-F, CGGAAGCTTGTTCTACCAGCAGCCTTGC (HindIII)

p71A12-R, GCTCTAGATTCTTGAATATTGCTCATGTATGAAAG (XbaI)

pMYB51-F, ACACACCTGCAGTGTACTAAAGAACTACTGTAA (PstI)

pMYB51-R, ACACACGTCGACCCATGGTCTTGATTCTTCAAACTTAGCT (SalI-NcoI)

pWRKY11-F, ACACACCTGCAGCTTCCCCACCCATATATAGCCA (PstI)

pWRKY11-R, ACACACGTCGACCCATGGGATGATTTCTTGGTCTGAGGAT (SalI-NcoI)

pAT5G25260-F, GCTCTAGACATAAAGTTGTAGTAAGAC (XbaI)

pAT5G2520-R, TTCCCGGGTTGAACATGTCTAGGATC (SmaI)

pPEN2-F, GCTCTAGAIGGACTAGCAAGGAATATC (XbaI)

pPEN2-R, AAGGCCTCTTGTCTTGATTCAGAAG (StuI)

The *PEN3*$_{pro}$*:GUS* line was provided by Yuki Ichinose (Okayama University, Japan).

II.8. Mutant seed stocks

The following insertion lines were obtained from the Arabidopsis Biological Resource Center, Columbus, OH: *cyp81F2-1* (SALK_073776), *myb51-1* (SM_3_16332),

jin1-7 (SALK_040500), *efr-2* (SALK_068675), *bak1-3* (SALK_034523).

The *fls2* (SAIL_691_C04) line was obtained from Jeffrey Dangl (University of North Carolina at Chapel Hill, USA). The *cyp79B2cyp79B3* line was obtained from John Celenza (Boston University, USA) and the *cerk1-2* (GABI_kat 096F09) insertion line from Naoto Shibuya (Meiji University, Japan).

III. Results

III.1. MAMPs elicit a strong response in the roots

To determine whether *Arabidopsis* roots respond to MAMPs, and if so, in which cell types, *promoter:GUS* transgenic lines were generated for four marker genes (*CYP71A12*, *MYB51*, *WRKY11* and *AT5G25260*) that are upregulated in seedlings treated with Flg22 (Denoux et al., 2008). *CYP71A12* encodes a cytochrome P450, highly homologous to CYP71A13, which has been shown to catalyze the conversion of indole-3-acetaldoxime (IAOx) to indole-3-acetonitrile (IAN) during camalexin biosynthesis (Nafisi et al., 2007). MYB51 is a transcription factor essential for the regulation of indole-glucosinolate biosynthesis (Gigolashvili et al., 2007). The transcription factor WRKY11 is a negative regulator of basal resistance in *Arabidopsis* (Journot-Catalino et al., 2006). Finally, *AT5G25260* encodes a nodulin-like protein of unknown function that is an ortholog of the mammalian protein flotillin-1 involved in lipid raft formation.

All four GUS reporter genes were activated after Flg22 treatment in the elongation zone (EZ) of seedling roots (Figure 1A). This response was completely abolished in *fls2* and *bak1-3* mutants, lacking a functional Flg22 receptor (FLS2) (Gomez-Gomez et al., 2001) or an associated receptor kinase (BAK1) (Chinchilla, 2007), respectively (Figure 1B). Moreover, no induction was observed after treatment with a control Flg22 polypeptide derived from *Agrobacterium tumefaciens* that does not activate FLS2-mediated signaling (Figure 1C). Finally, the general kinase inhibitor K252a, which blocks FLS2 internalization (Robatzek et al., 2006) and impairs the FLS2-BAK1 (Chinchilla, 2007) interaction, and brefeldin A (BFA), which inhibits FLS2 recycling to

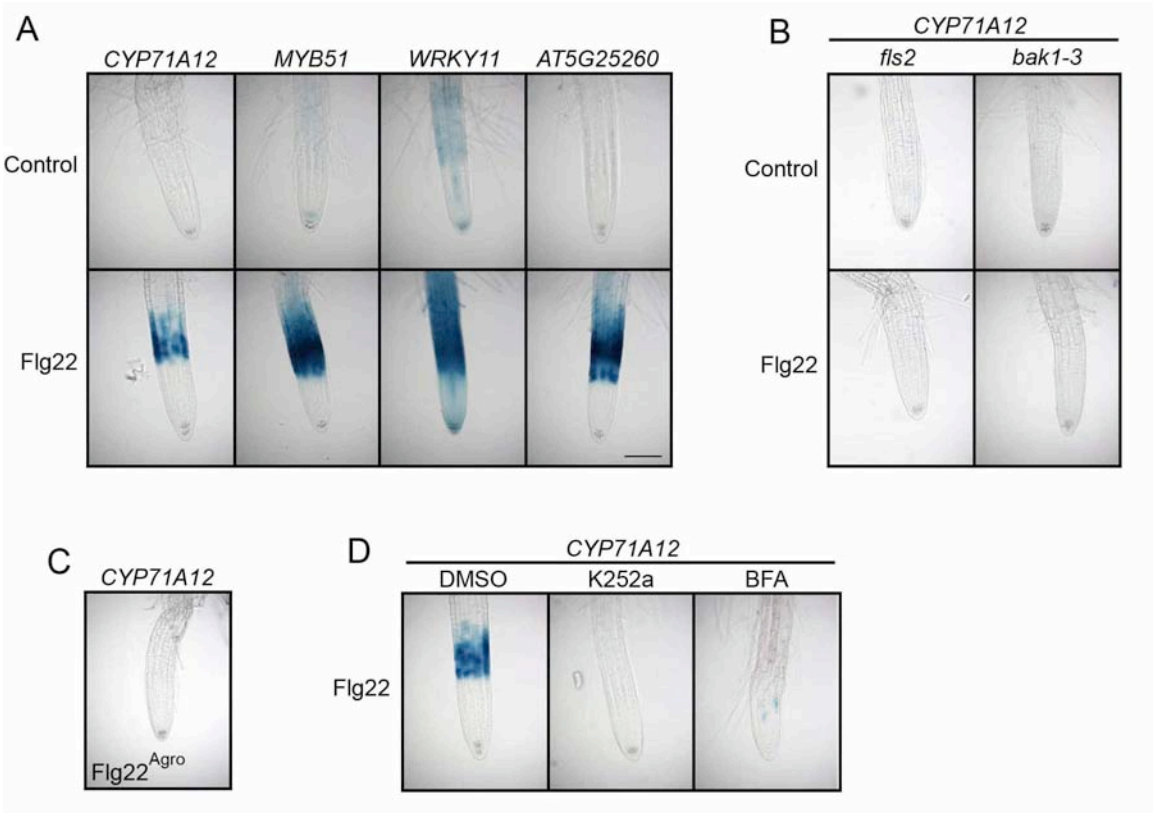

Figure 1. Flg22 elicits *promoter:GUS* reporter gene expression in transgenic *Arabidopsis* seedlings.

(A) Flg22 elicits expression of GUS reporter genes in the root elongation zone. Transgenic seedlings carrying *CYP71A12*$_{pro}$*:GUS, MYB51*$_{pro}$*:GUS*, *WRKY11*$_{pro}$*GUS,* or *AT5G25260*$_{pro}$*:GUS* reporters were treated with 100nM Flg22 for 3h (*MYB51* and *WRKY11*) or 5h (*CYP71A12* and *AT5G25260*) before GUS staining.

(B) Flg22-elicitation of a *CYP71A12*$_{pro}$*:GUS* depends on the Flg22 receptor FLS2 and the accessory receptor-like kinase BAK-1. Transgenic *fls2*;*CYP71A12*$_{pro}$*:GUS* or *bak1-3; CYP71A12*$_{pro}$*:GUS* seedlings were treated with 100nM Flg22 for 5h before GUS staining.

(C) A peptide corresponding to *Agrobacterium tumefaciens* flagellin does not activate *CYP71A12*$_{pro}$*:GUS.* Transgenic *CYP71A12*$_{pro}$*:GUS* seedlings were treated with 100nM Flg22^{Agro} for 5h before GUS staining.

(D) Flg22-elicitation of a *CYP71A12*$_{pro}$*:GUS* is blocked by the kinase inhibitor K252a and the membrane transport inhibitor brefeldin A (BFA). Transgenic *CYP71A12*$_{pro}$*:GUS* seedlings were co-treated with 100nM Flg22 plus 1% DMSO, 1μM K252a in DMSO or 100μg/mL BFA in DMSO for 5h before GUS staining.

the membrane (Robatzek et al., 2006), suppressed the Flg22 response in the roots (Figure 1D). The *MYB51*, *WRKY11* and *AT5G25260* reporters (but not the *CYP71A12* reporter), were also activated by Flg22 in seedling leaves (Figure S1).

Consistent with the conclusion that Flg22-elicited gene expression in roots is localized in the EZ, Flg22-elicited callose deposition, a well-studied response to MAMPs, was also localized in the root EZ (Figure 2). Similar to the GUS reporter assays results shown in Figure 1, callose deposition was completely abolished in *fls2* and *bak1-3* mutants. In the *pmr4-1* mutant that lacks a functional callose synthase, no callose deposition was observed as well.

Three additional MAMPs, peptidoglycan (PGN), Elf26, and chitin, were also tested for GUS reporter gene activation and callose deposition in *Arabidopsis* seedling roots. PGNs consist of a polymer of alternating *N*-acetylglucosamine and *N*-acetyl-muramic acid residues cross-linked by small peptides. PGN from *Bacillus subtilis*, a well-known root colonizer, strongly activated the *CYP71A12* and *MYB51* GUS reporters in the EZ, similar to the Flg22 response (Figure S2A), and activated the *WRKY11* and *At5G25260* promoters to a lesser extent. This response was not due to flagellin contamination of the PGN solution since the *fls2* mutant was still able to strongly respond to PGN (Figure S2B). Furthermore, the GUS response to PGNs was abolished in the *bak1-3* mutant (Figure S2C). PGNs also triggered callose deposition in the EZ. However, this latter response was much weaker and more variable than the Flg22-elicited response (Figure S3A). Elf26 did not activate any of the GUS reporters or callose deposition in the roots (Figures S2A; S3A). This was not due to a lack of activity of Elf26 since it did trigger callose deposition in wild-type cotyledons, but not in the Elf26 receptor mutant

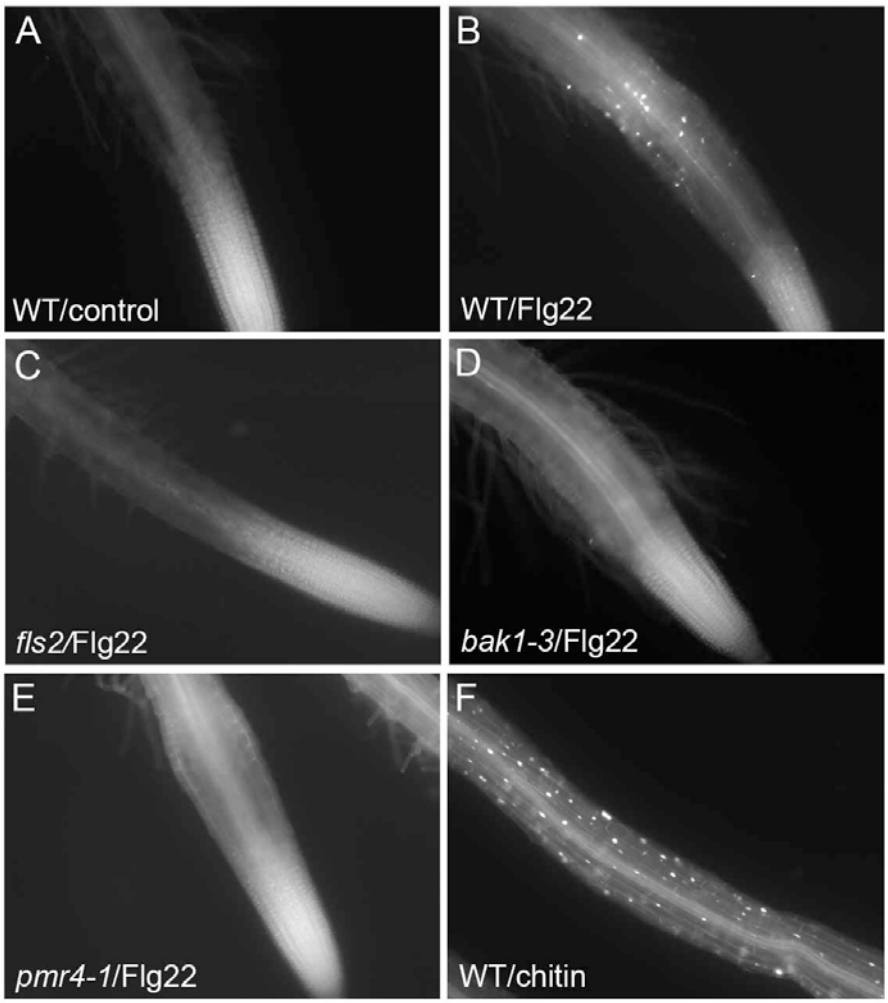

Figure 2. Flg22 and chitin elicit callose deposition in wild type and mutant *Arabidopsis* roots.
Callose staining in roots of seedlings treated with water **(A)**; 1μM Flg22 **(B-E)**; 500μg/mL chitin for 18h **(F)**. Col-0 **(A, B, F)**; *fls2* **(C)**; *bak1-3* **(D)***; pmr4-1* **(E)**.

efr-2 (Figure S3B). Finally, chitin, a sugar polymer of N-acetylglucosamine, triggered a strong root response, but in contrast to Flg22 and PGN, GUS reporter gene activation and callose deposition occurred throughout the entire mature zones of the roots (Figure 2F; S4), but not in the EZ. The chitin-elicited callose response was abolished in the *cerk1-2* mutant that is insensitive to chitin, as well as in the callose synthase mutant *pmr4-1* (Figure S5C; S5D). In contrast to Flg22 and PGN and consistent with chitin-elicited signaling in leaves (Shan et al., 2008), the response to chitin was independent of BAK1 since the *bak1-3* mutant had a normal GUS response and normal callose deposition following chitin treatment (Figure S4; S5E).

III.2. Indole glucosinolates are required for callose deposition in roots

Our laboratory previously reported that Flg22-elicited callose deposition in *Arabidopsis* cotyledons was dependent on the synthesis of indol-3-ylmethylglucosinolate (I3G) biosynthesis, which is in turn dependent on the transcription factor MYB51 (Clay et al., 2009). Callose deposition in cotyledons was also found to be dependent on the cytochrome P450 CYP81F2, required for the methoxylation of I3G to form 4-methoxy-I3G (4M-I3G), the PEN2 myrosinase, which presumably hydrolyzes 4M-I3G, and the PEN3 ABC transporter. Here, we observed that the Flg22-elicited callose deposition in roots was abolished in *myb51-1*, *cyp79B2cyp79B3* (impaired in I3G biosynthesis), *cyp81F2-1* or *pen2-1* mutants (Figure 3). Significantly, we observed the same results for chitin-elicited callose deposition (Figure S5). Interestingly, *PEN2* was activated by Flg22 in the EZ (Figure S6A). The PEN3 ABC transporter, required for the Flg22-elicited callose response in the cotyledons, was required for the chitin-elicited response

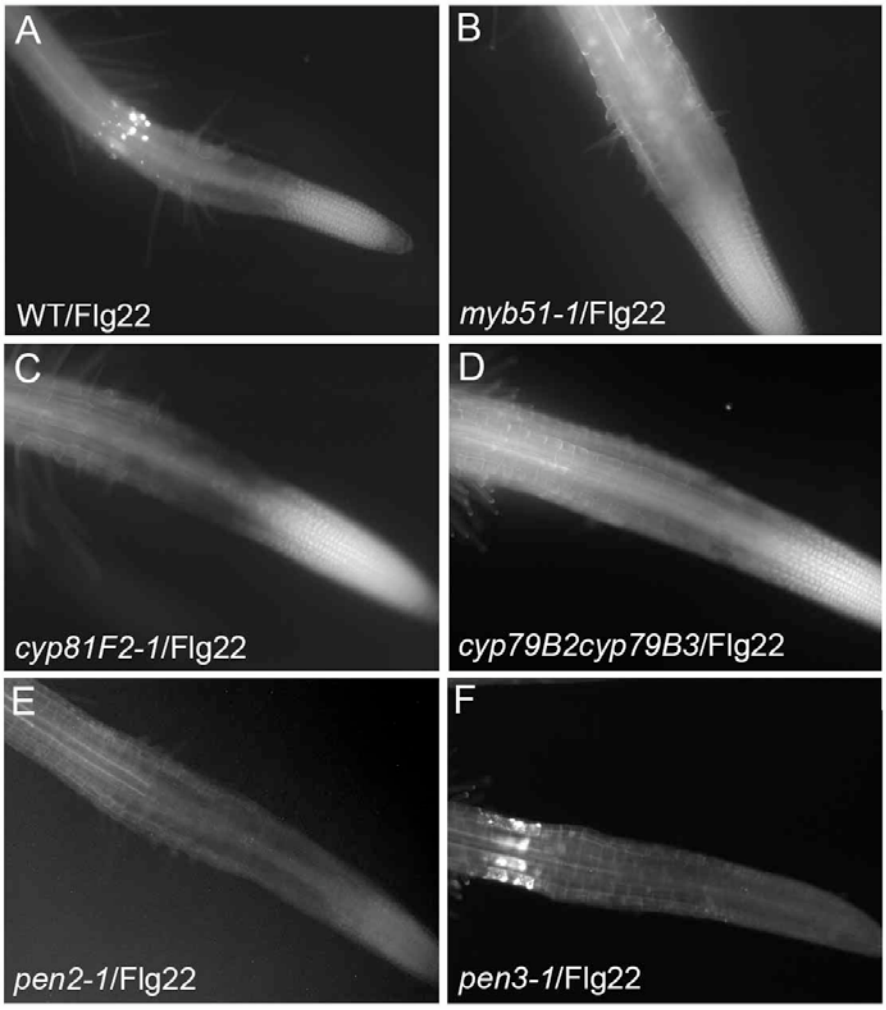

Figure 3. *MYB51*, *CYP81F2* and *PEN2* are required for Flg22-elicited callose deposition in roots.
Callose staining in the roots of Col-0 **(A)**; *myb51-1* **(B)**; *cyp81F2-1* **(C)**; *cyp79B2cyp79B3* **(D)**; *pen2-1* **(E)**; or *pen3-1* **(F)** treated with 1μM Flg22 for 18h.

but not the Flg22-elicited response in the roots (Figures 3F, S5O). This latter observation correlates with the expression pattern of *PEN3*$_{pro}$*:GUS* (Figure S6B). *PEN3* was expressed throughout the entire root except in the root tip. Moreover, *PEN3*$_{pro}$*:GUS* expression in roots was activated by chitin but not by Flg22 (Figure S6B). It is possible that another ABC transporter, expressed in the root EZ, is substituting for PEN3 after Flg22 elicitation in the roots.

III.3. Coronatine suppresses MAMP responses in the roots

To further study MAMP signaling activation in *Arabidopsis* roots, we tested if *Pseudomonas fluorescens* WCS417r, a root colonizer and ISR inducer, activated *CYP71A12*$_{pro}$*:GUS* in seedling roots. Intriguingly, despite the fact that *P. fluorescens* produces MAMPs, WCS417r did not activate the *CYP71A12* reporter (Figure 4A). We hypothesized that WCS417r may suppress MAMP responses in the roots. To test this hypothesis, seedlings were pre-inoculated with *P. fluorescens* WCS417r prior to Flg22 treatment. Indeed, WCS417r suppressed the Flg22-elicited activation of the *CYP71A12* reporter (Figure 4A) as well as the Flg22-elicited deposition of callose in the root EZ (Figure 4D). Although *Pseudomonas syringae* is generally considered to be a leaf pathogen, it is known to colonize roots (Bais et al., 2004). Similar to *P. fluorescens* WCS417r, *P. syringae* pv. *tomato* strain DC3000 (*Pst*DC3000) did not activate any of the four GUS reporters or the deposition of callose in the root EZ and suppressed the Flg22-elicited activation of the reporters and callose deposition (Figures 4B; 4D; S7). Similar results were obtained with *P. syringae pv. maculicola* strain ES4326 (*Psm*ES4326) for the *CYP71A12*$_{pro}$*:GUS* reporter (Figure S8A). These results suggest that both P.

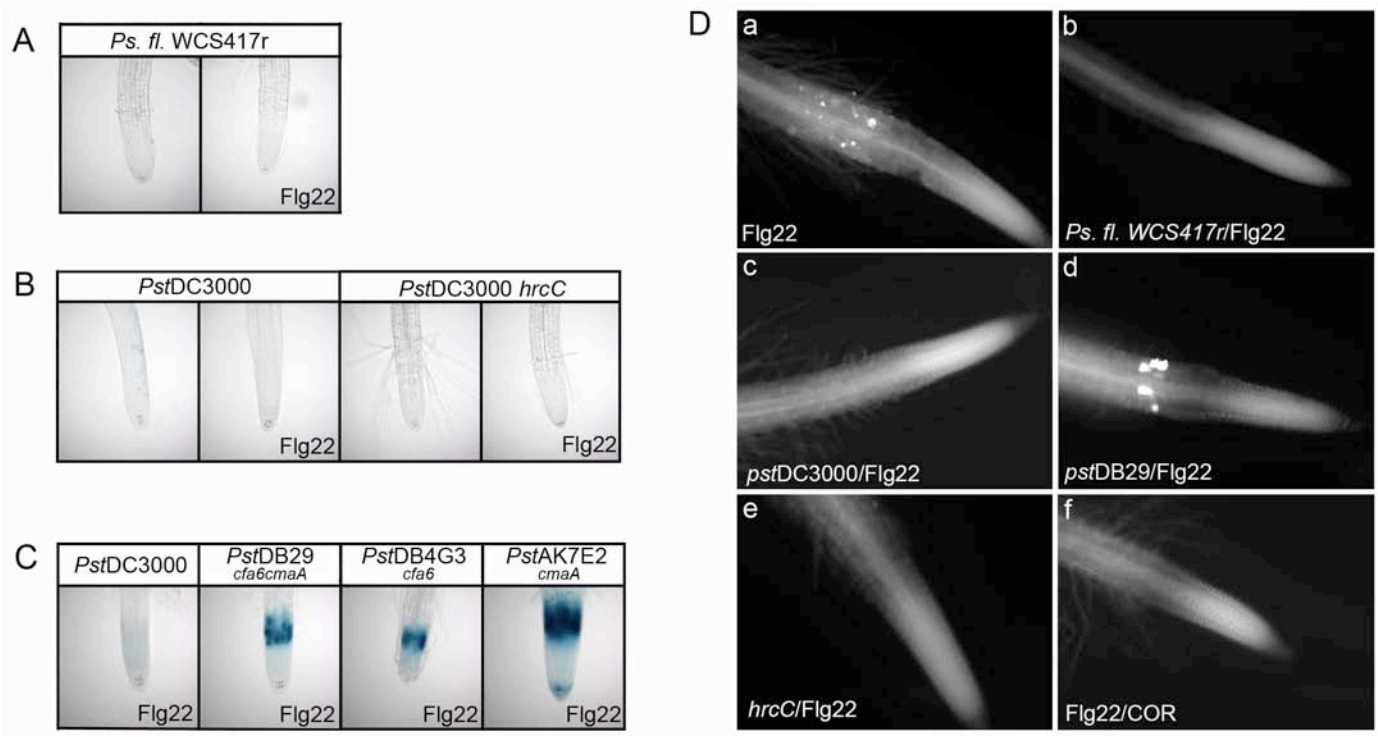

Figure 4. *P. syringae* and *P. fluorescens* suppress Flg22-elicited responses in *Arabidopsis* roots.
(A) *P. fluorescens* WCS417r suppresses Flg22-elicited expression of $CYP71A12_{pro}$*:GUS*. Transgenic $CYP71A12_{pro}$*:GUS* seedlings were pre-infected with WCS417r for 18h and then treated with 100nM Flg22 for 5 h before GUS staining.
(B) The *P. syringae* DC3000 Type III secretion system is not required for suppression of Flg22-elicited expression of $CYP71A12_{pro}$*:GUS*. Transgenic $CYP71A12_{pro}$*:GUS* seedlings were pre-infected with *Pst* DC3000 or *Pst* DC3000(*hrcC)* (CUCPB5112) for 18h and then treated with 100nM Flg22 for 5h before GUS staining.
(C) Coronatine synthesized by *P. syringae* DC3000 suppresses Flg22-elicited expression of $CYP71A12_{pro}$*:GUS*. Col-0 Seedlings were infected with *Pst* DC3000 or the coronatine deficient mutant DB29 (cfa^-; cma^-), DB4G3 (cfa^-) or AK7E2 (cma^-) for 18h. The collected media was filtered. Transgenic $CYP71A12_{pro}$*:GUS* seedlings were incubated in the filtered media and treated with 100nM Flg22 for 5h before GUS staining.
(D) Coronatine suppresses the Flg22-elicited deposition of callose in *Arabidopsis* roots. Col-0 seedlings treated with 1μM Flg22 for 18h **(a)** or pre-infected with *P. fl.* WCS417r **(b)** *Pst* DC3000 **(c)**, *Pst* DB29 (cfa^-; cma^-) **(d)**, or *Pst* CUCPB5112 ($hrcC^-$) **(e)** for 6h or co-treated with 1μM COR **(f)** and then treated with 1μM Flg22 for 18h.

fluorescens and *P. syrinage* actively suppress MAMP-elicited responses in *Arabidopsis* roots.

Two *P. syringae*-encoded mechanisms have been described that suppress MAMP-mediated responses, the injection of effectors directly into plant cells via the type three secretion system (TTSS) (He et al., 2006; Li et al., 2005) and the synthesis of the phytotoxin coronatine (COR) (Li et al., 2005; Melotto et al., 2006). A non-polar *hrcC* mutant of *Pst*DC3000, CUCPB5112, which is unable to inject its type three effectors, suppressed both Flg22-elicited $CYP71A12_{pro}$*:GUS* reporter gene expression and callose deposition (Figures 4B; 4D), indicating that the suppression is independent of the TTSS. These results contrast with previous studies that showed that various *P. syringae* TTSS effectors suppress Flg22-induced callose deposition in leaves (Hauck et al., 2003; Kim et al., 2005). Consistent with published data, *Pst*DC3000 but not *Pst*DC3000 *hrcC* suppressed Flg22-elicited callose deposition in seedling cotyledons (Figure S9).

In contrast to the *hrcC* mutants, several COR deficient mutants of *Pst*DC3000 (Figure 4C) and the COR deficient *cfa6* mutant of *Psm*ES4326 (Figure S8A) failed to block Flg22-elicited GUS reporter gene activation in the root EZ. Furthermore, the COR deficient *Pst*DC3000 DB29 mutant did not suppress Flg22-elicited callose deposition (Figure 4D). These results show that COR synthesis is required for suppression of the Flg22-elicited responses in the root EZ. To determine whether COR is sufficient to suppress these responses, seedlings were co-treated with Flg22 and purified COR. In the absence of bacteria, COR suppressed the Flg22-elicited GUS and callose responses (Figures 4D; 5; S10). COR also suppressed activation of the GUS reporters by PGN and chitin (Figure S2; S4), as well as chitin-elicited callose deposition (Figure S11B).

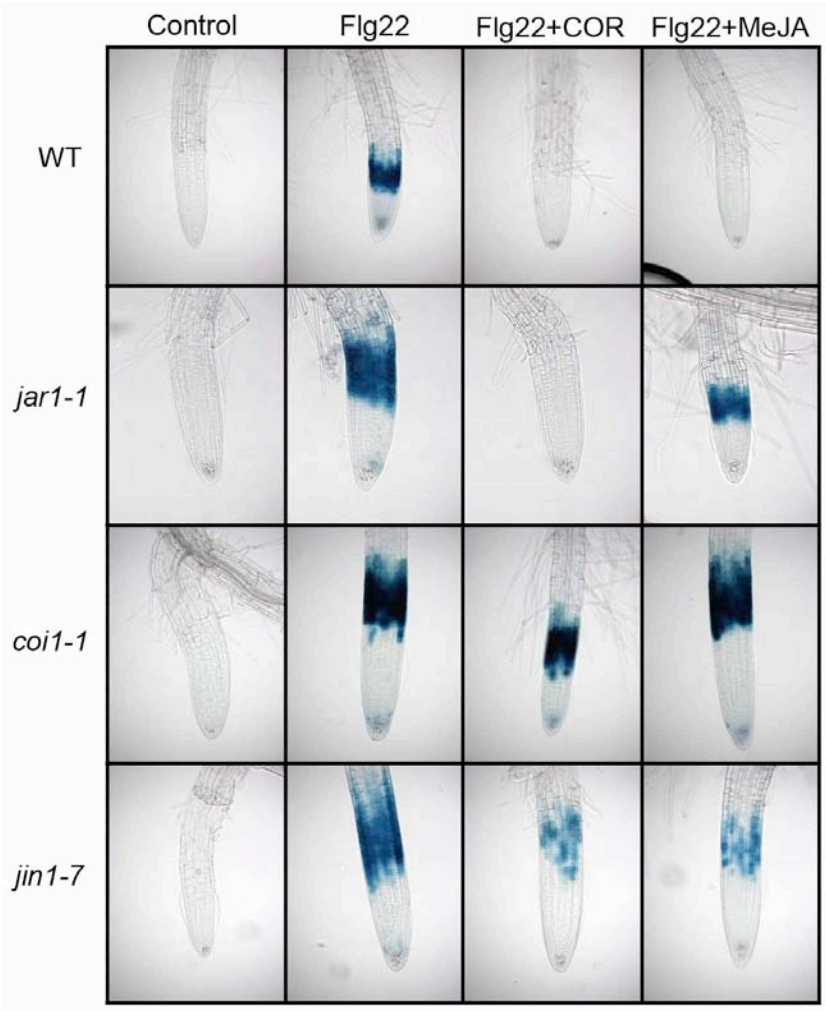

Figure 5. COR acts as a mimic of JA-Ile in suppressing Flg22-elicited expression of *CYP71A12*$_{pro}$*:GUS* in *Arabidopsis* roots.
Transgenic seedlings carrying a *CYP71A12*$_{pro}$*:GUS* reporter construct in WT, *jar1-1*, *coi1-1,* or *jin1-7* backgrounds were co-treated with 1μM COR or 10μM MeJA and 100nM Flg22 for 5h before GUS staining.

However, because PGN alone elicited relatively low levels of callose deposition, it was difficult to determine whether COR suppressed this response. *E. coli* was unable to suppress the Flg22-elicited activation of *CYP71A12*$_{pro}$*:GUS* in the roots showing that the ability to suppress the MAMP response in roots is not shared by all bacteria. Consistent with the fact that Flg22 derives from the *Pseudomonas aeruginosa* flagellin, the *P. aeruginosa* strain PA14 activated the *CYP71A12*$_{pro}$*:GUS* reporter in the absence of Flg22 (Figure S8A; S8B).

COR is a polyketide composed of two parts, coronafacic acid (CFA) and coronamic acid (CMA) linked by an amide bond. To test which component of COR is necessary for the suppressive effect on innate immunity, the *Pst*DC3000 *cor*$^{-}$ mutants DB4G3, deficient in CFA, AK7E2, deficient in CMA, and DB29, deficient in both CFA and CMA, were tested for their ability to suppress the Flg22 response in the roots. All three mutants failed to suppress the *CYP71A12*$_{pro}$*:GUS* reporter response (Figure 4C), suggesting that intact COR is required for suppression. Since the bacterial strains used in these experiments were isogenic and grew at a similar rate, it is highly unlikely that the lack of suppression of the *cor*$^{-}$ mutants was due to a non-specific growth defect.

Although COR is known to be a chlorosis-inducing toxin, the following observations make it unlikely that COR is blocking MAMP-activated responses simply because of its toxic effect on roots. First, no visible cell damage was observed in the roots by microscopic observation after COR treatment. Second, COR did not affect the expression of other GUS-reporters expressed in the root tip, such as *DR5:GUS* (data not shown), or in the mature zone of the root, such as *PEN3*$_{pro}$*:GUS* (Figure S6B).

III.4. The MAMP response in the roots suppressed by COR is independent of SA signaling

SA signaling plays a major role in *Arabidopsis* resistance to *P. syringae* and the mutual antagonism between the SA and JA signaling pathways is well documented. It is generally accepted that COR, similarly to JA-Ile, suppresses the SA pathway. We reasoned that if COR suppresses MAMP-activated signaling as a consequence of JA-SA antagonism, then MAMP-mediated signaling pathways should be dependent on SA signaling. To test this hypothesis, two mutants in the SA pathway (*sid2-2*, *npr1-1*) were tested for their callose response to MAMPs in roots. SID2 is an isochorismate synthase required for the production of SA (Wildermuth et al., 2001). NPR1 is a key regulator of many SA-responsive genes and is required for the SA-mediated systemic acquired resistance (Cao et al., 1994; Cao et al., 1997). Neither *sid2-2* nor *npr1-1*, however, was impaired in Flg22 and chitin-elicited callose deposition in the EZ (Figures 6A; S5). These SA-related mutants were also crossed with the four *promoter:GUS* reporter lines. Flg22-elicited activation of the GUS reporters was similar to wild type seedlings in the *sid2-2* and *npr1-1* mutants (Figure 6B). In addition, treatment of seedlings with exogenous SA, did not activate the $CYP71A12_{pro}$*:GUS* reporter or trigger callose deposition (Figure 6A; 6C). Altogether, these results show that the response to MAMPs in the roots, and by extension, its suppression by COR, are independent of SA signaling.

We next investigated if SA signaling was able to antagonize COR. Because COR is believed to act as a JA mimic, we predicted that treating the seedlings with exogenous SA should impair, at least partially, the COR-mediated suppression of MAMP-signaling. Although a 6h pre-treatment of the seedlings with SA did not block COR-mediated

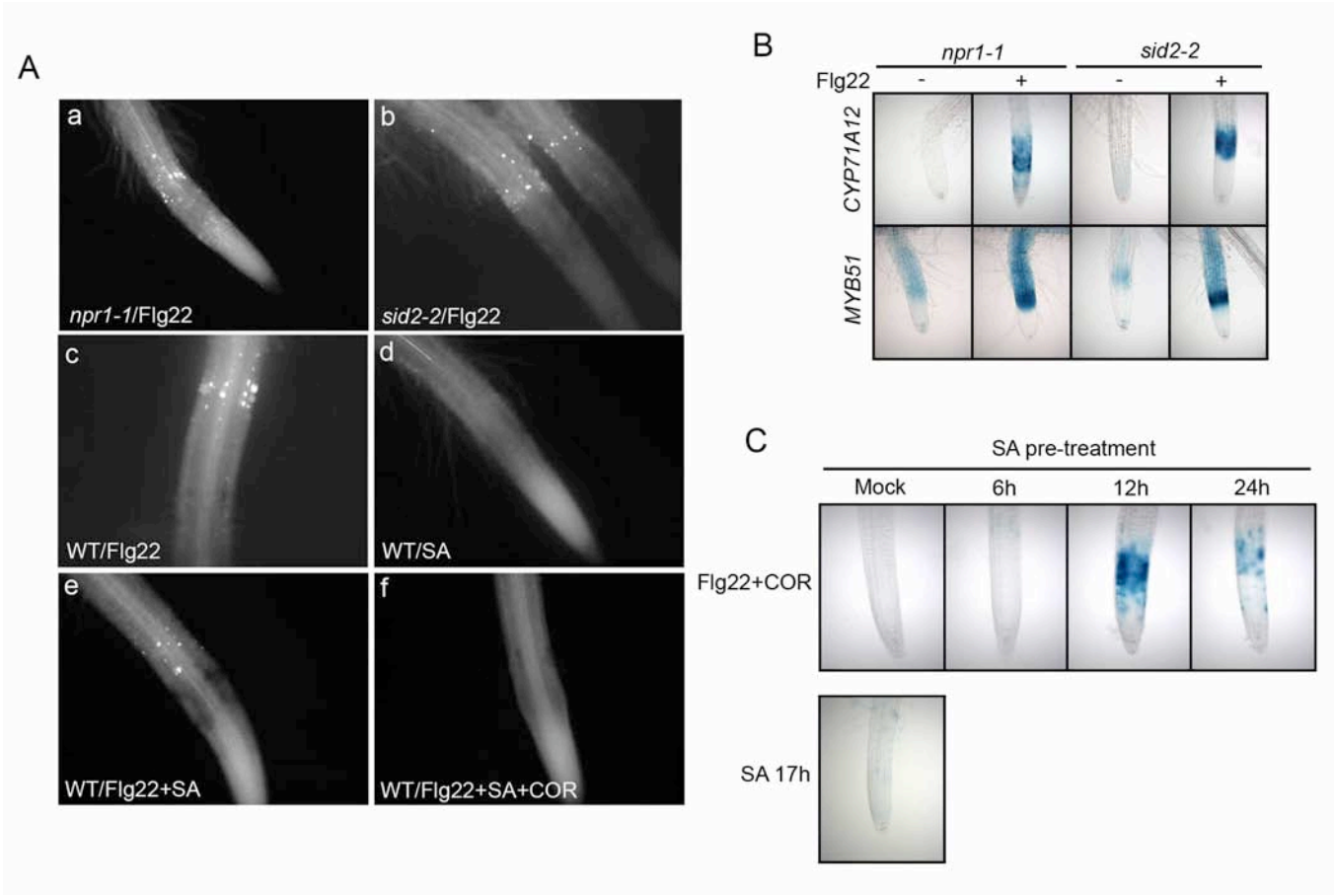

Figure 6. COR-mediated suppression of Flg22-elicited signaling is independent of SA signaling.
(A) Callose deposition in the roots of *Arabidopsis* seedlings. *npr1-1* **(a)**; *sid2-2* **(b)** or Col-0 **(c-f)** seedlings treated with 1μM Flg22 for 18h **(a, b, c)**. Wild-type seedlings were treated with 100μM SA for 24h **(d)** or pre-treated with 100μM SA for 6h and then treated with 1μM Flg22 with or without 1μM COR for 18h **(e,f)**.
(B) Flg22 elicited $CYP71A12_{pro}$*:GUS* or $MYB51_{pro}$*:GUS* expression in *Arabidopsis* seedlings. $CYP71A12_{pro}$*:GUS* or $MYB51_{pro}$*:GUS* seedlings were treated with 100nM Flg22 for 3h (for *MYB51*) or 5h (for *CYP71A12*) in *npr1-1* or *sid2-2* mutant backgrounds.
(C) $CYP71A12_{pro}$*:GUS* seedlings were pre-treated with 100μM SA for 6, 12 or 24 hours and then treated with 100nM Flg22 and 1μM COR for 5h.

suppression of Flg22-elicited callose deposition or suppression of *CYP71A12$_{pro}$:GUS* (Figure 6A, 6C), a 12h pre-treatment with SA was able to block COR-mediated suppression of the *CYP71A12$_{pro}$:GUS* reporter (Figure 6C). These data show that under the appropriate conditions, SA is able to antagonize COR-mediated suppression, consistent with a recent report that showed that the kinetics of SA-signaling plays a major role in the suppression of JA responses (Koornneef et al., 2008). Importantly, these data do not contradict the experiments described above showing that COR-mediated suppression of MAMP responses does not appear to be a consequence of antagonism between the JA and SA signaling pathways.

III.5. ET signaling potentiates the MAMP response in roots and antagonizes the COR suppressive effect

After ruling out a major role for SA signaling in the MAMP-mediated activation of root defense responses, we sought to determine the role of other hormone signaling pathways. Our laboratory has recently shown that ET signaling plays a key role in Flg22-elicited callose deposition in cotyledons (Clay et al., 2009). The ET signaling mutant *ein2-1* was impaired in Flg22-elicited activation of the *CYP71A12$_{pro}$:GUS, MYB51$_{pro}$:GUS* and *WRKY11$_{pro}$:GUS* reporters (Figure 7A) in roots. Flg22-elicited activation of *MYB51* and *CYP71A12* were also analyzed by qRT-PCR in wild-type and *ein2-1* roots. The levels of *MYB51* and *CYP71A12* transcripts were lower in the Flg22-treated *ein2-1* roots compared to wild-type roots, confirming the importance of ET signaling (Figure 7B). However, the basal expression level of these genes was also lower in *ein2-1* mutant roots and a significant activation by Flg22 was observed for both genes,

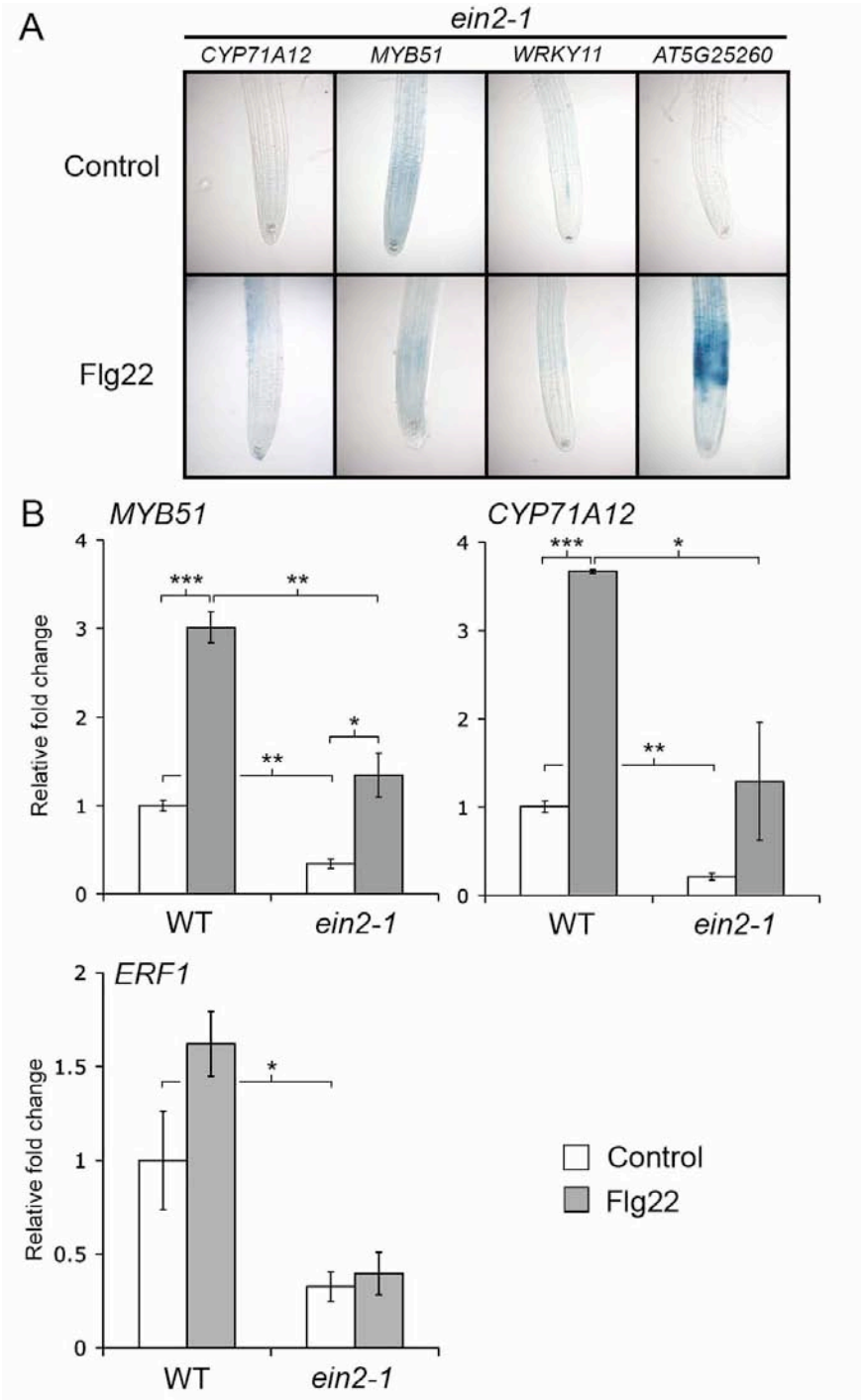

Figure 7. Flg22-elicited gene expression in Arabidopsis roots is potentiated by ET signaling.
(A) Transgenic seedlings carrying *CYP71A12*$_{pro}$*:GUS, MYB51*$_{pro}$*:GUS, or AT5G25260*$_{pro}$*:GUS* reporters in an *ein2-1* mutant background were treated with 100nM Flg22 for 3h (for *MYB51*) or 5h (for *CYP71A12* or *ATG25260*) before GUS staining.
(B) qRT-PCR analysis of *MYB51*, *CYP71A12* and *ERF1* transcript levels in the roots of 2-week old Col-0 or *ein2-1* seedlings grown on vertical plates and treated with 1μM Flg22 for 3h.
In **(B)** and **(C)**, qRT-PCR data represent the mean ±SD of 3 replicate samples. *P<0.05, **P<0.01, ***P<0.001; two tailed *t* test.

even though GUS staining driven by *MYB51*$_{pro}$*:GUS* and *CYP71A12*$_{pro}$*:GUS* was not observed. A lower basal expression of *MYB51* was also observed in *ein2-1* cotyledons compared to wild-type by looking at the *MYB51*$_{pro}$*:GUS* reporter line (Figure S1). These data indicate the existence of a Flg22-elicited ET-independent signaling pathway for the activation of *MYB51* and *CYP71A12*.

The qRT-PCR results shown in Figure 7B suggest that a low level of Flg22-elicited activation of *MYB51* and *CYP71A12* occurs in *ein2-1* roots and that under appropriate staining conditions it should be possible to observe a low level of GUS activity for the two reporters in *ein2-1* seedlings. Indeed, a weak activation of *MYB51*$_{pro}$*:GUS* and *CYP71A12*$_{pro}$*:GUS* in the *ein2-1* mutant background was detected by staining overnight instead of 4h (Figure S12). The absence of ET signaling in the roots of the *ein2-1* mutant seedlings was confirmed by qRT-PCR analysis of *ERF1*, whose expression is known to be *ein2*-dependent (Lorenzo et al., 2003) (Figure 7B). Additional evidence for the existence of a Flg22-activated ET-independent pathway came from analysis of the *AT5G25260*$_{pro}$*:GUS* reporter line. Unlike *MYB51*$_{pro}$*:GUS*, *CYP71A12*$_{pro}$*:GUS* and *WRKY11*$_{pro}$*:GUS*, *AT5G25260*$_{pro}$*:GUS* was strongly activated by Flg22 in the *ein2-1* mutant (Figure 7A). Taken together, these data show that *MYB51* and *CYP71A12* can be activated by both ET-dependent and ET-independent signaling pathways.

The results described above led us to hypothesize that Flg22 triggers a modest response by an ET-independent pathway but that ET signaling significantly potentiates this response. This was confirmed by examining callose deposition in the roots of various ET signaling mutants. *ein2-1*, *etr1-3* and *ein3-1* mutants were compromised for

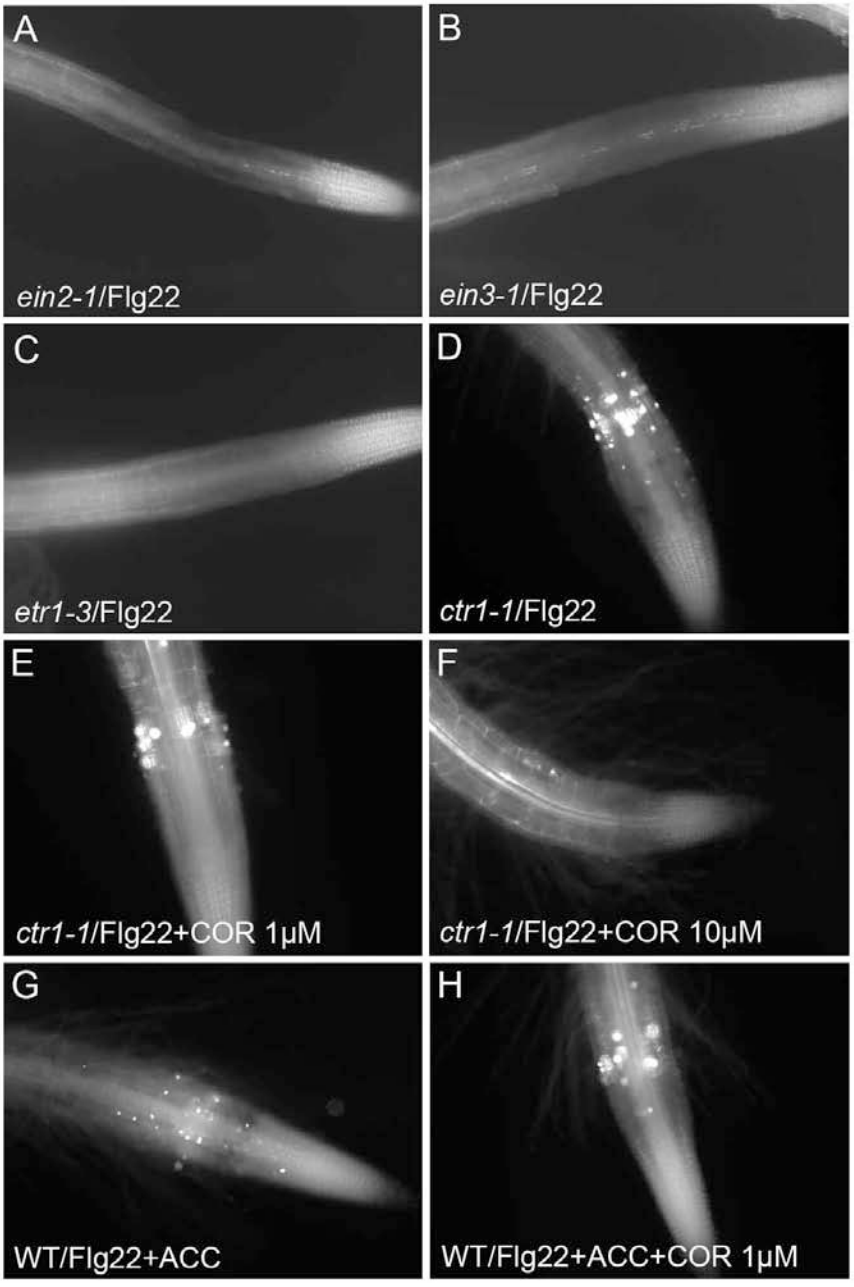

Figure 8. The Flg22-elicited callose response in the roots is ethylene-dependent.
Callose staining in the roots of *ein2-1* **(A)**, *ein3-1* **(B)**, *etr1-3* **(C)**, *ctr1-1* **(D, E, F)** or Col-0 **(G, H)** seedlings treated with 1μM Flg22 **(A-D)**; 1μM Flg22 and 1μM COR **(E)** or 1μM Flg22 and 10μM COR **(F)** for 18h. Wild-type seedlings were pre-treated with 100μM ACC for 24h and then treated with 1μM Flg22 with or without 1μM COR **(G, H)**.

both Flg22 and chitin-elicited callose deposition in the roots (Figure 8; S5), showing that ET signaling is necessary for detectable callose deposition. Furthermore, the Flg22-elicited callose response was much stronger in the constitutive ET signaling mutant *ctr1-1* and a 10 fold higher concentration of COR (10 μM) was required to obtain reproducible suppression of the callose response in this mutant (Figures 8E; 8F). In addition, COR, at the concentration that normally suppresses the callose response in wild-type seedlings, was ineffective when seedlings were pre-treated with ACC, the precursor of ET (Figure 8H). ACC alone did not trigger the activation of any GUS reporters (data not shown) or callose deposition (Figure S5P) in the root EZ. These results are consistent with the observations that the ET overproducing mutant *eto1-1* and the constitutive ET-signaling pathway mutant *ctr1-1* do not constitutively produce callose in the root EZ (data not shown). Taken together, the data show that ET signaling potentiates the MAMP response and by doing so, antagonizes the COR suppressive effect.

III.6. COR represses the ET-dependent and ET-independent transcriptional activation of MAMP-responsive genes in roots

Because our data show that ET signaling potentiates MAMP signaling in the roots (Figures 7; 8), we sought to determine whether COR blocks the transcriptional activation of key ET-dependent genes involved in MAMP signaling. Among the ET-dependent responses required for MAMP-induced callose deposition in *Arabidopsis* cotyledons and roots is MYB51-dependent biosynthesis of I3G. As shown in Figure 7B, Flg22 activates *MYB51* by both ET-dependent and ET-independent mechanisms and the activation of this gene is potentiated by ET. Monitoring expression of *MYB51* by qRT-PCR showed that it

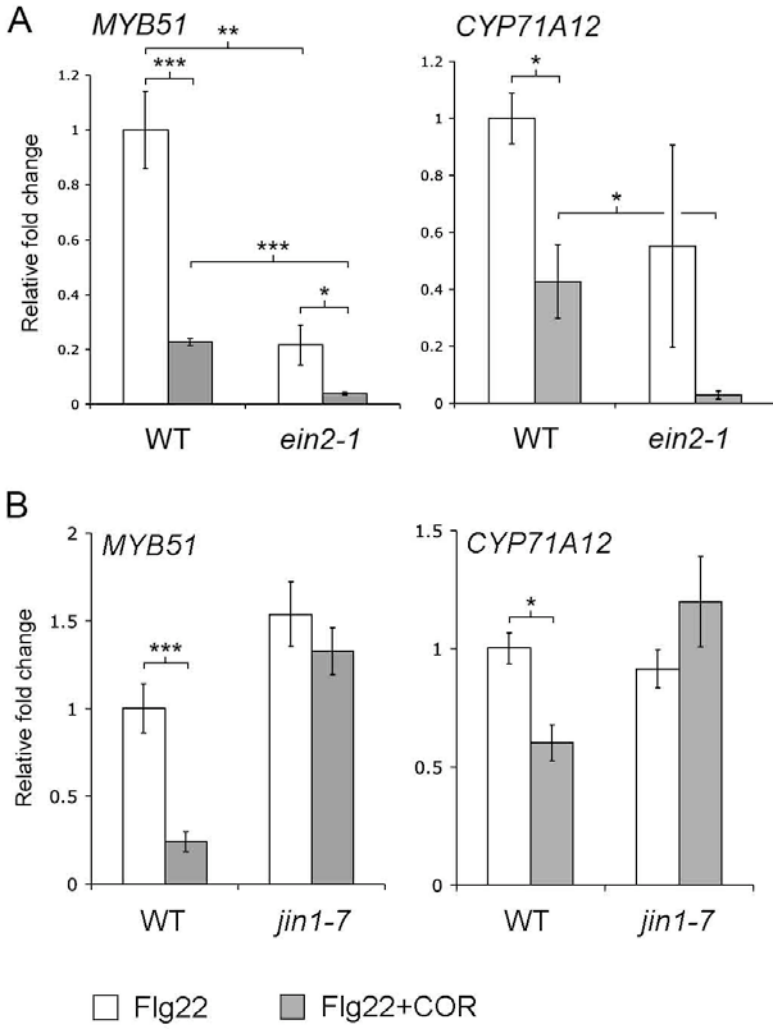

Figure 9. COR suppresses both the ET-dependent and ET-independent Flg22-elicited activation of *MYB51*, *CYP71A12* and requires MYC2.
qRT-PCR analysis of *MYB51* and *CYP71A12* transcript levels in the roots of 2 week old Col-0, *ein2-1* **(A)** or *jin1-7* **(B)** seedlings grown on vertical plates and treated with 1μM Flg22 with or without 0.2μM COR for 3h.

is repressed by COR (Figure 9). This result was confirmed by examining the *MYB51*$_{pro}$*:GUS* transgenic line treated with Flg22 and COR (Figures S10). The fact that *AT5G25260* activation by Flg22 is ET-independent (Figure 7A) and that COR is able to repress the expression of this gene (Figure S10) suggested that COR blocks both the ET-dependent and ET-independent pathways activated by MAMPs. We therefore examined the expression of *MYB51* and *CYP71A12* in *ein2-1* roots after treatment with Flg22 and COR. COR repressed *MYB51* and *CYP71A12* expression in the *ein2-1* mutant (Figure 9A) showing that COR suppresses both the ET-dependent and independent pathways.

III.7. COR acts through COI1 and MYC2 to suppress the response to MAMPs

As discussed above, COR is believed to act by mimicking JA-Ile. In accordance with this result, methyl-jasmonate (MeJA), also suppressed the Flg22 response in the roots in both the GUS reporter and callose deposition assays, although at a 10 times higher concentration than COR (Figures 5; 10C; S10). MeJA also suppressed chitin-elicited callose deposition (Figure S11C). To test if the COR/MeJA suppressive effect of the Flg22 response in roots is dependent on the canonical JA signaling pathway, different mutants impaired in JA signaling were tested. JA-amino acid conjugates such as JA-Ile bind to the E3 ubiquitin ligase COI1. COI1 is a major component of JA signaling and *coi1* mutants are severely impaired in multiple JA responses. The binding of JA-Ile to COI1 promotes the downstream interaction of JAZ proteins (for Jasmonate ZIM-domain) with the SCF^{COI1} ubiquitin ligase complex and their targeting to the proteasome. JAZ proteins are known to be repressors of the transcription factor JIN1 (for Jasmonate INsensitive 1), also known as MYC2. JIN1/MYC2 is a transcription factor involved in

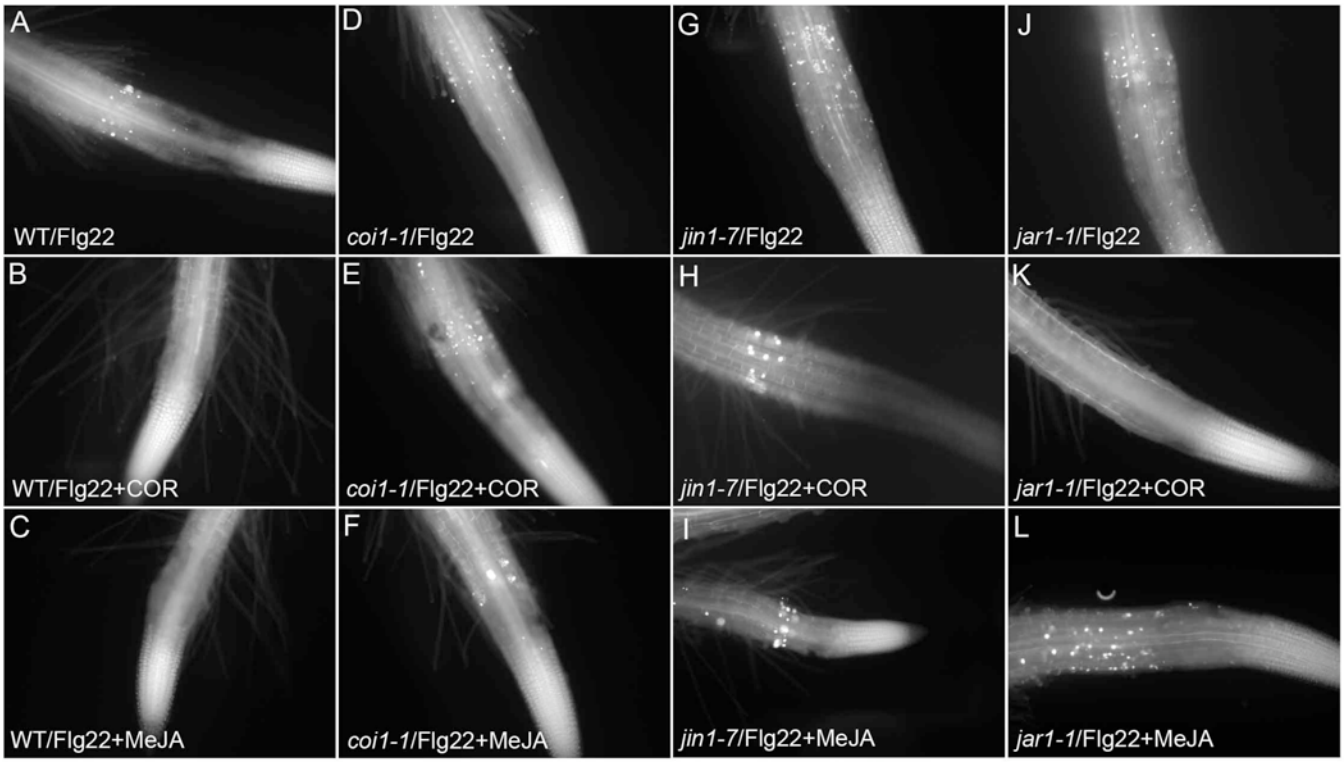

Figure 10. The COR-mediated suppression of the Flg22-elicited callose deposition in roots requires COI1 and MYC2.
Callose staining in the roots of Col-0 **(A, B, C)**; *coi1-1* **(D, E, F)**; *jin1-7* **(G, H, I)**; and *jar1-1* **(J, K, L)** treated with 1μM Flg22 **(A, D, G, J)**; 1μM Flg22 and 1μM COR **(B, E, H, K)**; or 1μM Flg22 and 10μM MeJA **(C, F, I, L)** for 18h.

many jasmonate responses. Among other phenotypes, *jin1/myc2* mutants are partially impaired in JA and COR-mediated root growth inhibition and are more susceptible to herbivorous insects such as *Helicoverpa armigera* (Dombrecht et al., 2007).

COR and MeJA were not able to suppress the Flg22-elicited GUS and callose deposition responses in the roots of *coi1-1* mutant (Figures 5; 10; S10). The *jin1-7* mutant was also impaired for both COR and MeJA-mediated suppression (Figures 5; 10; S10). However, a weak suppressive effect was detectable in the case of the GUS assay suggesting the existence of at least one additional "JIN1/MYC2-like" gene. The repression of *MYB51* and *CYP71A12* by COR after activation by Flg22 was also tested by qRT-PCR in *jin1-7* mutants. COR was unable to repress those genes in *jin1-7* roots confirming the essential role of *JIN1/MYC2* in the COR-mediated suppression of MAMP responses in roots (Figure 9B). JAR1 (for Jasmonic Acid Resistant 1) is an amino acid conjugase required for the formation of JA-Ile. It is believed that the conjugated form of JA is the actual signaling molecule because *jar1* mutants are resistant to JA, especially with respect to its root growth inhibitory effect. Interestingly, COR was still able to suppress the Flg22 response in the *jar1-1* mutant whereas MeJA did not (Figure 5; 10; S10). This result confirms that MeJA needs to be conjugated to an amino acid to be active and suppress the Flg22 response and that COR functions as a JA-Ile mimic downstream of JAR1. Significantly, the same results were obtained for COR and MeJA suppression of the chitin-elicited callose deposition in *coi1-1*, *jar1-1* and *jin1-7* (Figure S11).

IV. Discussion

Using sensitive and relatively high throughput assays to study MAMP signaling in *Arabidopsis* roots, we demonstrated that MAMPs elicit transcriptional responses and callose deposition in MAMP-specific locations. Flg22 and PGN trigger a response that is localized to the elongation zone (EZ) of the root tip. Most root pathogens initiate infection at the EZ, probably because the remodeling of cell walls that occurs in the EZ makes the cells more susceptible to pathogen attack. The EZ is also generally considered to be a major site of exudation of secondary metabolites. It is possible that these exudates attract pathogens by chemotaxis and that plants in turn evolved MAMP signaling in the EZ to trigger the deposition of callose and the exudation of antimicrobials. Unlike Flg22 and PGN, however, chitin elicits a response in the mature zones of roots but not in the root tips, including the EZ. This raises the interesting hypothesis that plants evolved tissue-specific innate immune responses to different MAMPs that depend on the nature of the attacking microorganism. Pathogenic rhizobacteria, unlike fungi and nematodes, generally cannot directly penetrate the epidermal layers of roots and therefore exploit the weakest part of the roots and potential wounds to attack. This is the case of *Ralstonia Solanacearum* that preferentially infects at the EZ and at the natural openings present at the junctions between the main and lateral roots (Vasse, 1995). Unlike bacteria, root pathogenic fungi and nematodes, both producing chitin, are able to successfully penetrate the epidermal layer and are generally able to infect throughout the entire root.

The localization of the Flg22 response in the EZ is not due to the localization of the Flg22 receptor in the EZ since FLS2 was shown to be expressed in the entire root (Robatzek et al., 2006). Another explanation could be that FLS2 is only internalized at the EZ. In preliminary experiments, however, in contrast to cotyledons, we could not detect any FLS2 internalization in the roots using an FLS2-GFP transgenic line. Another difference that distinguishes Flg22 and PGN from chitin is that Flg22 and PGN-elicited responses both require the accessory leucine-rich-repeat receptor-like kinase BAK1 (Figures 1B; 2D; S2C; S5E). This result, consistent with published reports (Shan et al., 2008) suggests that the pattern recognition receptor corresponding to PGN is probably associated with BAK1 and is most likely a LRR-RLK like FLS2. BAK1 was shown to be highly expressed in roots but its precise localization is not known. The Flg22 and PGN responses at the EZ might be explained by the potential EZ localization of BAK1. However, a transgenic line carrying a functional *35S-BAK1-GFP* construct showed normal callose deposition and no ectopic callose deposition outside of the EZ after Flg22 elicitation (data not shown). These results suggest that BAK1 localization is not responsible for the Flg22 and PGN responses localization.

Despite the differences in the localization of the responses and BAK1 dependency with respect to Flg22 and PGN, on the one hand, and chitin, on the other, we showed that most of the MAMP signaling pathway leading to callose deposition is conserved between Flg22 and chitin and between roots and leaves. Common features of Fgl22- and chitin-elicited signaling pathways include the requirement of ET signaling, MYB51-dependent I3G biosynthesis, CYP81F2-dependent 4-methoxylation of I3G, and the involvement of the PEN2 myrosinase. One difference that we observed between the Flg22 and chitin

responses, however, is that the ABC transporter PEN3, required for Flg22-elicited callose deposition in cotyledons, is required for the chitin-elicited callose deposition, but is not required for Flg22-elicited one. Therefore, it is possible that another ABC transporter is substituting for PEN3 in the EZ of root tip. PEN3 belongs to the PDR ABC transporter subfamily, which consists of 15 homologues. Examining Flg22-elicted callose deposition in the corresponding ABC transporter gene mutants may identify the PDR ABC transporter substituting for PEN3 in the EZ.

Similar to leaves, we showed that *P. syringae* is able to suppress MAMP induced callose deposition in roots. However, unlike the MAMP response in leaves, we demonstrated that *P. syringae*-mediated suppression is independent of the *P. syringae* TTSS, but dependent on the production of the phytotoxin COR, a structural mimic of the signaling molecule JA-Ile. COR is known to block root growth, which raised the concern that COR is suppressing the MAMP-activated responses in the EZ by just stopping root growth. This hypothesis was discarded since other root growth inhibitors such as auxin did not block the Flg22 response (data not shown). Moreover, cutting the meristematic zone of the root tip did not block the response to Flg22 (data not shown). Finally, Flg22 itself is known to block root growth (Gomez-Gomez and Boller, 2000). In fact, COR-mediated suppression of MAMP responses is dependent on the ubiqutin ligase COI1, a key regulator of JA signaling, similar to what was found for COR suppression of MAMP-induced stomatal closure in *Arabidopsis* leaves (Melotto et al., 2006). In addition, we demonstrated that COR-mediated suppression of the MAMP response in roots is also mostly dependent on the transcription factor MYC2 (also referred to as JIN1). However, we observed that COR-mediated suppression of MAMP signaling is also partially

independent of MYC2. A weak COR-mediated suppressive effect was detected in the *jin1-7* mutant in the case of the *CYP71A12$_{pro}$:GUS* reporter. That is, the activation of the *CYP71A12$_{pro}$:GUS* reporter by Flg22 was not completely suppressed by COR in the *jin1-7* background. Moreover, we found that *CYP81F2* activation by Flg22 in the roots is also repressed by COR but that the COR-mediated repression of *CYP81F2* is independent of MYC2 (i.e., it is not affected in a *jin1-7* mutant) (data not shown). These results, in parallel with the fact that the *jin1-7* mutant is only partially impaired for its COR-mediated root growth inhibition (Dombrecht et al., 2007), suggest the existence of at least one additional gene that could share a redundant function with MYC2.

Importantly, we found that COR is suppressing MAMP-elicited responses in the roots independently of the well-documented JA-SA antagonism. This result differs from the model generally accepted for the mode of action of COR based on the antagonism between JA and SA signaling. For example, the MAMP-elicited stomatal closure suppressed by COR requires the SA biosynthetic enzyme SID2 and the SA regulatory protein NPR1. Moreover, stomatal closure is induced by SA. COR is also believed to promote bacterial growth and persistence in plant tissue by taking advantage of the JA-SA antagonism since the growth of COR deficient mutants of *P. syringae* is restored to wild-type levels in the *Arabidopsis* mutants *sid2* and in the transgenic line *nahG,* unable to accumulate SA during infection. However, we found that the MAMP responses in roots that are suppressed by COR are SA-independent. This result correlates with the repression of the Flg22-induced *Arabidopsis* gene *NHO1* by COR (Li et al., 2005). Indeed, *NHO1* is activated by the non-host bacteria *P. phaseolicola* independently of SA signaling since the transgenic line *nahG* shows a normal activation of *NHO1* compared to

wild-type plants (Kang et al., 2003). Our data confirm that COR is able to suppress some SA-independent plant defense mechanisms.

The suppression of the MAMP responses in roots is not restricted to pathogens. Indeed, the beneficial bacterium *P. fluorescens* WCS417r also suppresses the Flg22 response in the roots. This result is counterintuitive since beneficial rhizobacteria are believed to protect the roots against potential pathogens by inducing plant defense. However, it is possible that the suppression of MAMP signaling is necessary for successful root colonization by PGPRs. In addition, the fact that *P. fluorescens* WCS417r suppresses MAMP signaling in the roots also contradicts the prevailing view that MAMPs are the molecular determinants responsible for ISR. It is possible that the early phases of root colonization by plant growth promoting bacteria requires the suppression of MAMP signaling to protect the bacteria against MAMP-elicited antimicrobial exudates. Once the colonization is achieved, however, the bacteria may be protected against the plant antimicrobials by the formation of a biofilm and stop the suppression of MAMP signaling allowing ISR. To our knowledge, *P. fluorescens* does not produce coronatine or compounds with related structures. Therefore, it is likely that this bacterium suppress the MAMP-response in roots via a different mechanism.

One possible mechanism by which *P. fluorescens* suppresses MAMP signaling in roots may relate to the fact that MAMP signaling is largely ET dependent. As shown in Figures 7 and 8, ET significantly potentiates the response to Flg22 in roots and this potentiating role of ET is necessary to observe detectable levels of callose. A role for ET as an important modulator of plant defense responses has also been described in many previous studies. In particular, ET was shown to increase the expression of the SA-

marker gene *PR1* in response to SA (Lawton et al., 1994). In addition, ET was shown to modulate NPR1-mediated crosstalk between SA and JA (Leon-Reyes et al., 2009). Interestingly, the *P. fluorescens* genome encodes an ACC deaminase, which degrades ACC, the ET precursor, into 2-oxobutyrate and ammonia. The ACC deaminases of beneficial rhizobacteria have been shown to play a positive role in plant growth and colonization of roots by other beneficial microorganisms such as arbuscular Mycorrhizas (Belimov et al., 2009; Gamalero et al., 2008; Wang et al., 2000). It is possible that beneficial microbes use this enzyme to decrease ACC levels and ET production in roots, thereby suppressing the MAMP response and allowing them to colonize the root surface. However, the role of MAMP signaling in plant growth promoting bacteria root colonization needs to be addressed. Studying the expression of the ACC deaminase and the suppression of MAMP signaling at different stages of *P. fluorescens* root colonization could provide us with a better understanding of the mechanisms involved in root colonization and ISR. A systematic approach combining the *promoter:GUS* lines and the assays described in this paper with mutants transposon libraries of various root colonizing bacteria, pathogenic or beneficial, will help us determine the strategies that different bacteria have evolved to suppress MAMP-elicited responses in roots.

Supplementary figures

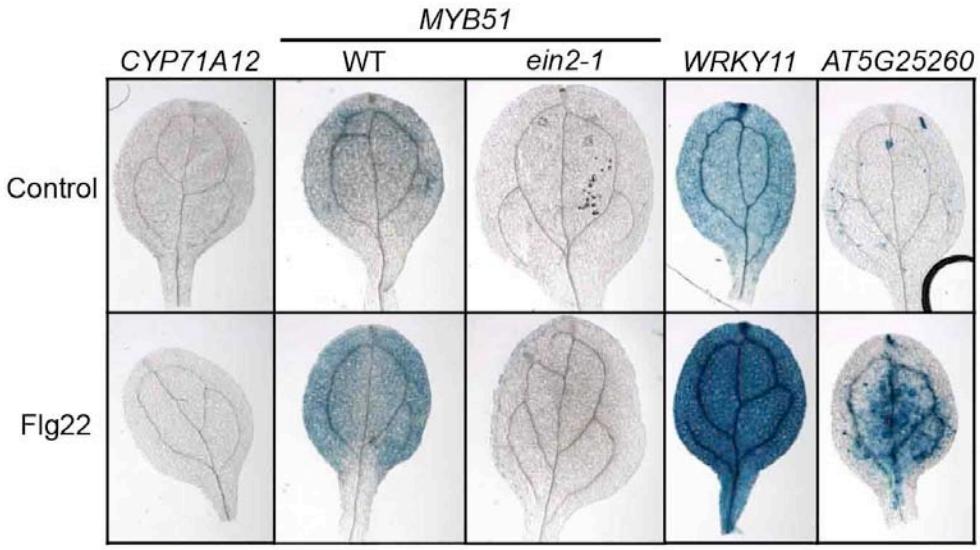

Figure S1. Activation of the promoter:GUS reporters in cotyledons.
Transgenic seedlings carrying *CYP71A12*$_{pro}$*:GUS, MYB51*$_{pro}$*:GUS* (in WT or *ein2-1* background), *WRKY11*$_{pro}$*GUS,* or *AT5G25260*$_{pro}$*:GUS* reporters were treated with 100nM Flg22 for 3h (*MYB51* and *WRKY11*) or 5h (*CYP71A12* and *AT5G25260*) before GUS staining.

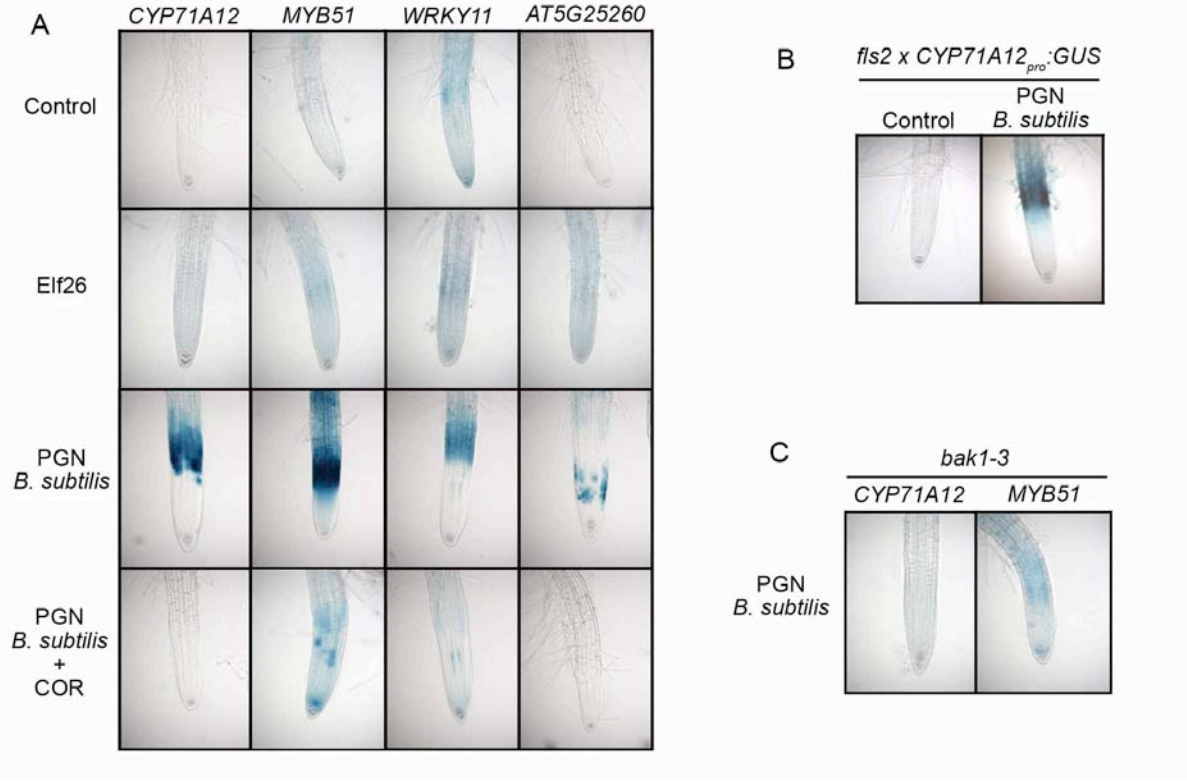

Figure S2. GUS staining in the roots of *promoter:GUS* reporters after PGN or Elf26 treatment.
(A) Transgenic seedlings carrying *CYP71A12*$_{pro}$*:GUS*, *MYB51*$_{pro}$*:GUS*, *WRKY11*$_{pro}$*GUS,* or *AT5G25260*$_{pro}$*:GUS* reporters were treated with 1μM Elf26 or 100μg/mL *B. subtilis* PGN with or without 1μM COR for 5 hours before GUS staining.
(B) *fls2* seedlings carrying a *CYP71A12*$_{pro}$*:GUS* reporter were treated with 100μg/mL *B. subtilis* PGN for 5 hours before GUS staining.
(C) *bak1-3* seedlings carrying *CYP71A12*$_{pro}$*:GUS* or *MYB51*$_{pro}$*:GUS* reporters were treated with 100μg/mL *B. subtilis* for 5 hours before GUS staining.

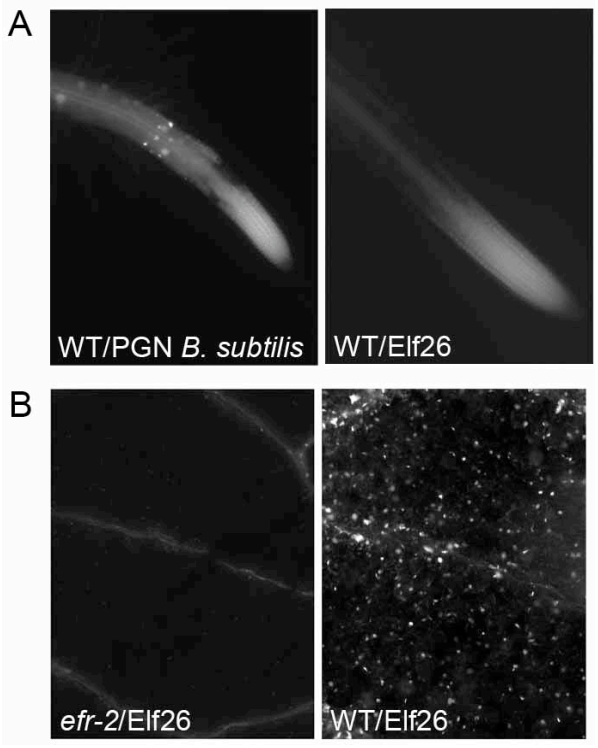

Figure S3. Callose staining in Arabidopsis seedling roots after PGN or Elf26 treatment.
(A) Callose staining in the roots of Col-0 seedlings treated with 100μg/mL PGN or 1μM Elf26 for 18h.
(B) Callose staining in the cotyledons of Col-0 and *efr-2* seedlings treated with 1μM Elf26 for 18h.

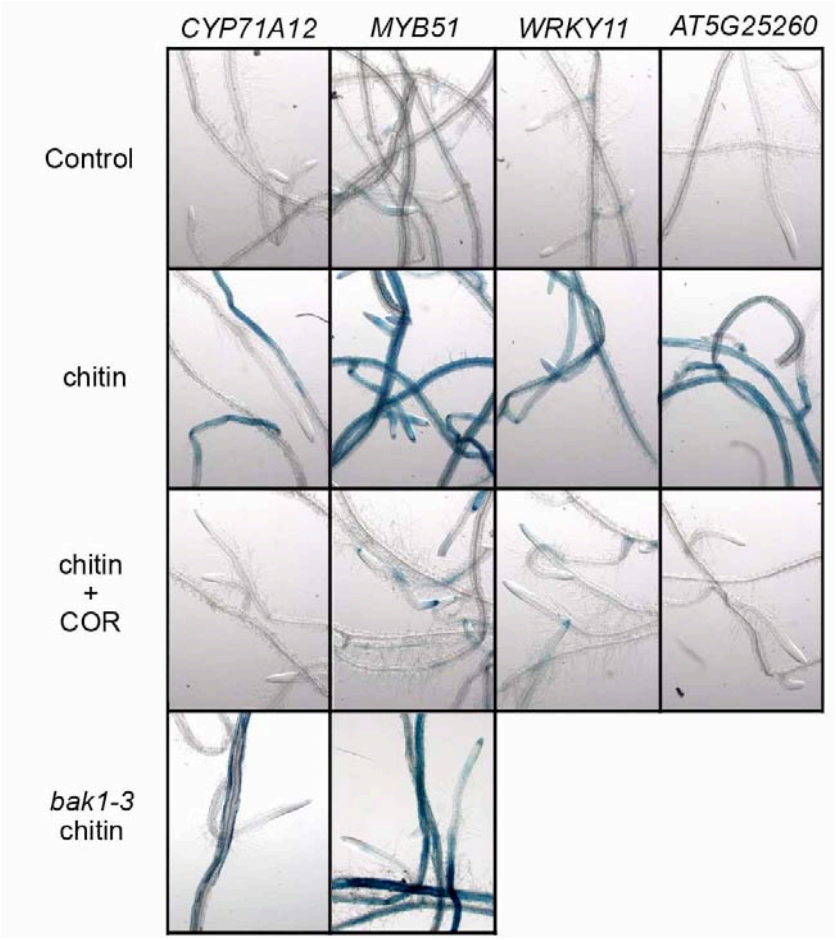

Figure S4. GUS staining in the roots of *promoter:GUS* reporters after chitin treatment. Transgenic seedlings carrying *CYP71A12*$_{pro}$*:GUS*, *MYB51*$_{pro}$*:GUS*, *WRKY11*$_{pro}$*GUS,* or *AT5G25260*$_{pro}$*:GUS* reporters in wild-type or *bak1-3* mutant backgrounds were treated with 100µg/mL chitin with or without 1 µM COR for 20 hours before GUS staining.

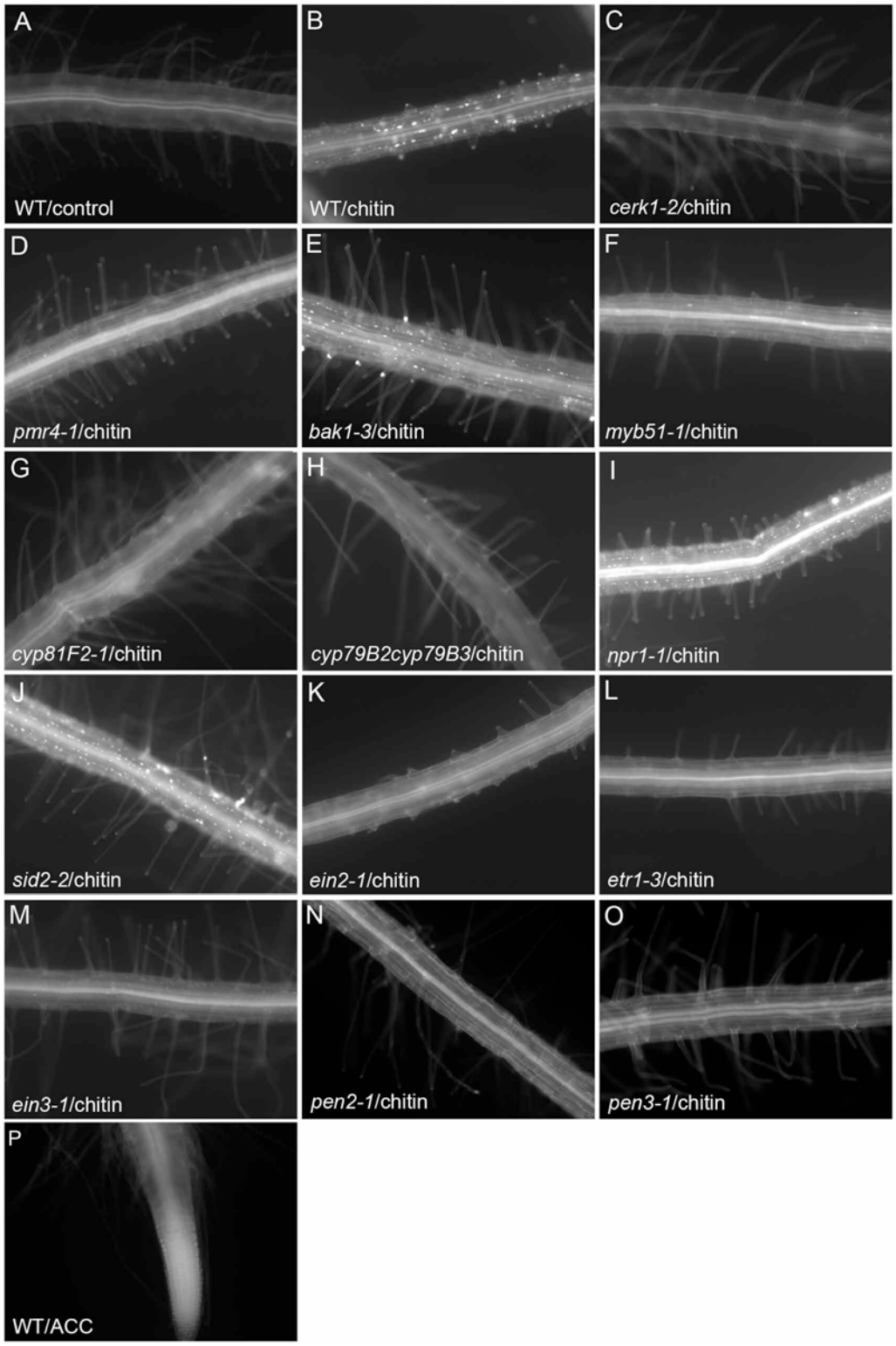

Figure S5. Callose staining in seedling roots of various *Arabidopsis* mutants after chitin and ACC treatment.

Callose staining in seedling roots treated with 500μg/mL chitin **(A-O)** for 18h or 100μM ACC for 24h **(P)**. Col-0 **(A, B, P)**; *cerk1-2* **(C)**; *pmr4-1* **(D)**; *bak1-3* **(E)**; *myb51-1* **(F)**; *cyp81F2-1* **(G)**; *cyp79B2cyp79B3* **(H)**; *npr1-1* **(I)**; *sid2-2* **(J)**; *ein2-1* **(K)**; *etr1-3* **(L)**; *ein3-1* **(M)**; *pen2-1* **(N)**; *pen3-1* **(O)**.

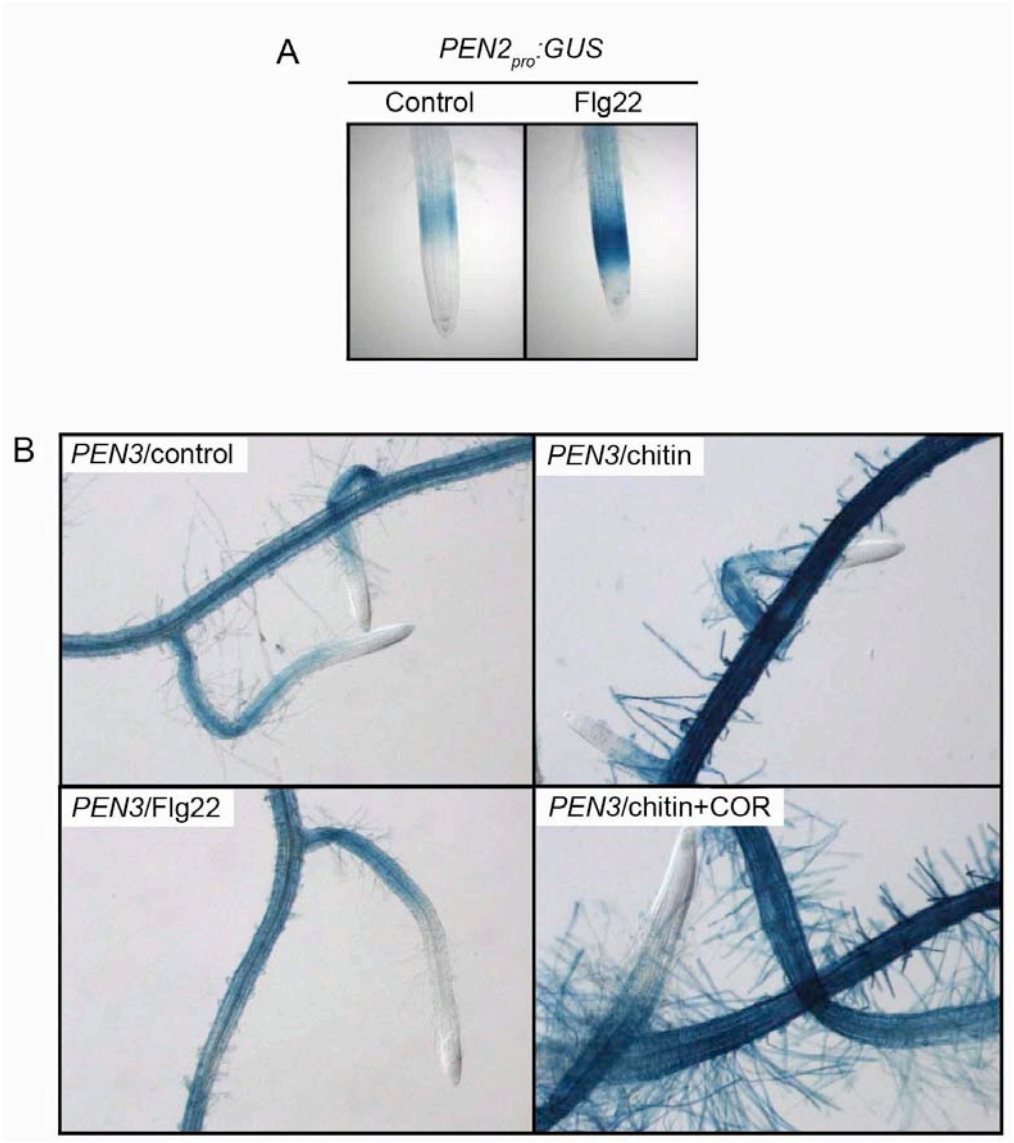

Figure S6. GUS staining in the roots of *PEN2* and *PEN3 promoter:GUS* reporters. Transgenic seedlings carrying $PEN2_{pro}$*:GUS* **(A) or** $PEN3_{pro}$*:GUS* **(B)** reporters treated with 1μM Flg22 for 6h or 100μg/mL chitin for 20h with or without 1μM COR before GUS staining.

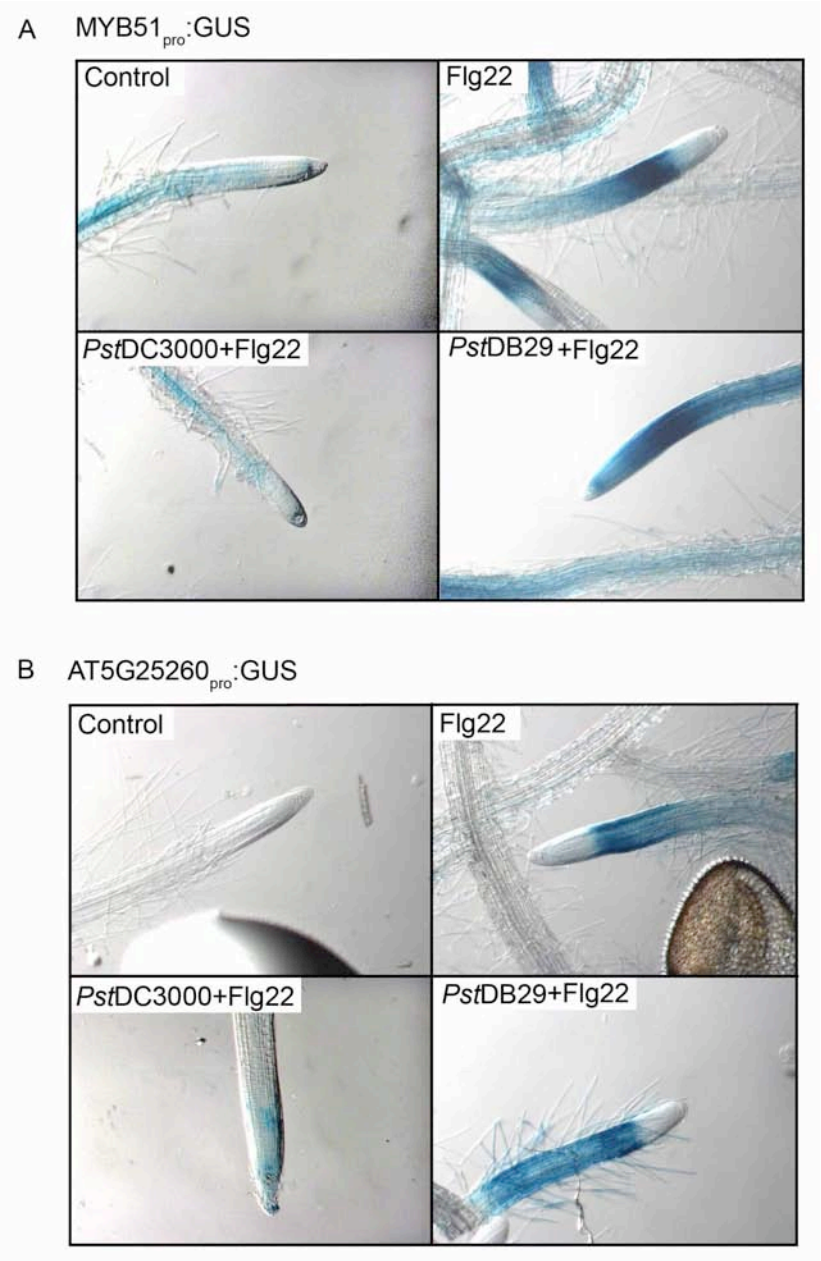

Figure S7. GUS staining in seedling roots of *promoter:GUS* reporters after pre-infection with *Pst* DC3000 or *Pst* DB29 (*cfa*$^-$; *cma*$^-$) followed by Flg22 treatment.
Col-0 Seedlings were infected with *Pst* DC3000 or the coronatine deficient mutant *Pst* DB29 (*cfa*$^-$, *cma*$^-$) for 18h. The collected media was filtered. *MYB51*$_{pro}$*:GUS* **(A)** or *AT5G25260*$_{pro}$*:GUS* **(B)** seedlings were incubated in the filtered media and treated with 100nM Flg22 for 3h (for *MYB51*) or 5h (for *AT5G25260*) before GUS staining.

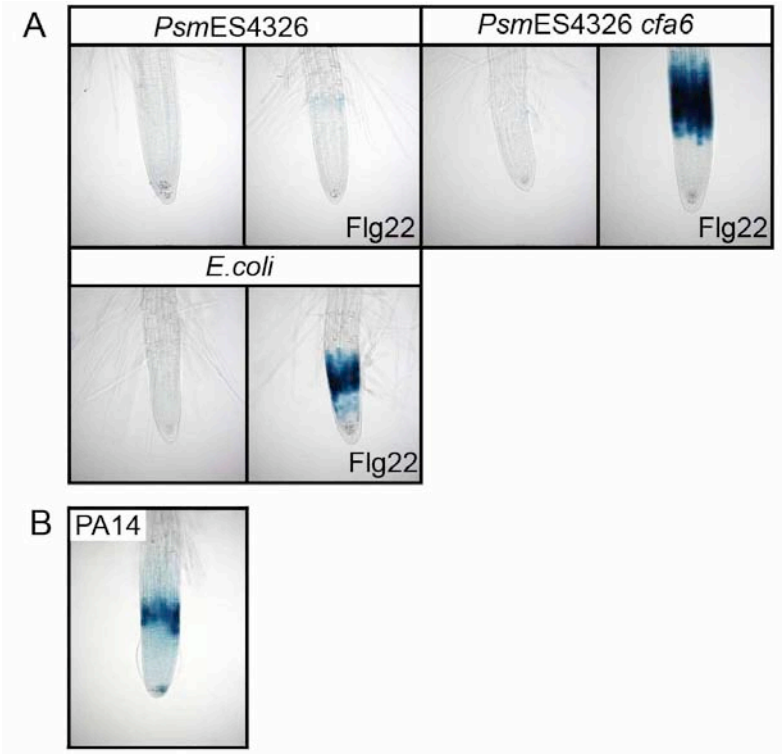

Figure S8. GUS staining in the roots of transgenic *CYP71A12$_{pro}$:GUS* seedlings after pre-infection with various bacteria followed with or without Flg22 treatment..
(A) *CYP71A12$_{pro}$:GUS* transgenic seedlings pre-infected by *Psm* ES4326, *Psm* ES4326-*cfa6*, or *E. coli* DH5α for 18h and then treated with 100nM Flg22 for 5h before GUS staining.
(B) *CYP71A12$_{pro}$:GUS* seedlings infected with *P. aeruginosa* strain PA14 for 24h.

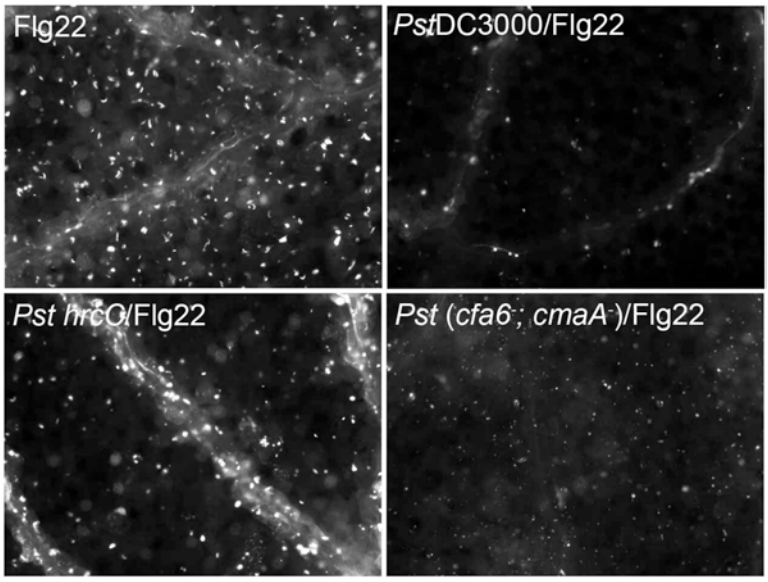

Figure S9. Flg22-elicited callose deposition in cotyledons of Col-0 seedlings after infection with *P. syringae hrcC* and coronatine deficient mutants.
Col-0 seedlings were pre-infected with *Pst* DC3000, *Pst* DB29 (*cfa*$^-$; *cma*$^-$) or *Pst* CUCPB5112 (*hrcC*$^-$) for 6h and then treated with 1μM Flg22 for 18h.

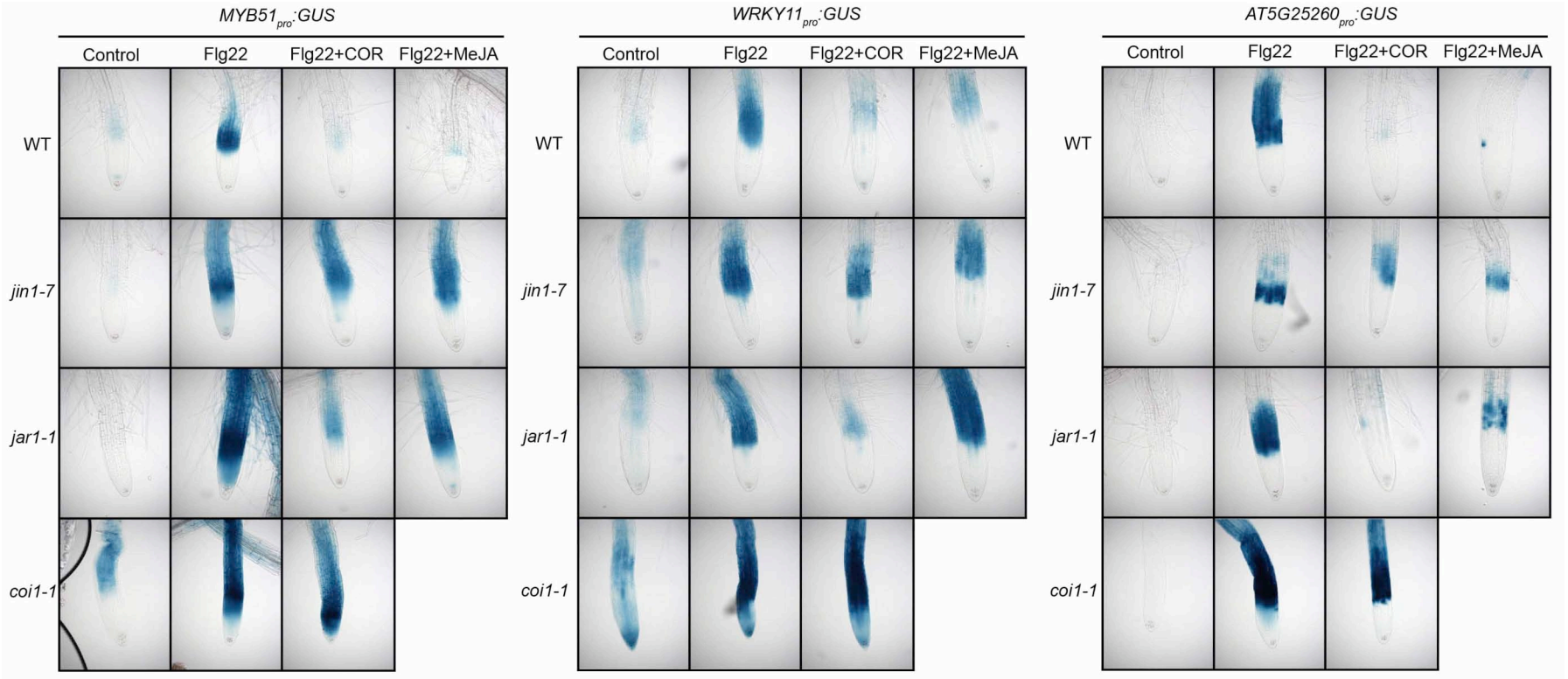

Figure S10. GUS staining in *Arabidopsis promoter:GUS* seedlings after Flg22 + COR or Flg22 + MeJA treatments in Col-0, *coi1-1*, *jin1-7* and *jar-1* seedlings.

Transgenic seedlings carrying $MYB51_{pro}$*:GUS*, $WRKY11_{pro}$*GUS,* or $AT5G25260_{pro}$*:GUS* reporters in WT, *jar1-1*, *coi1-1* or *jin1-7* backgrounds were co-treated with 1μM COR or 10μM MeJA and 100nM Flg22 for 3h (for *MYB51* and *WRKY11*) or 5h (for *AT5G25260*).

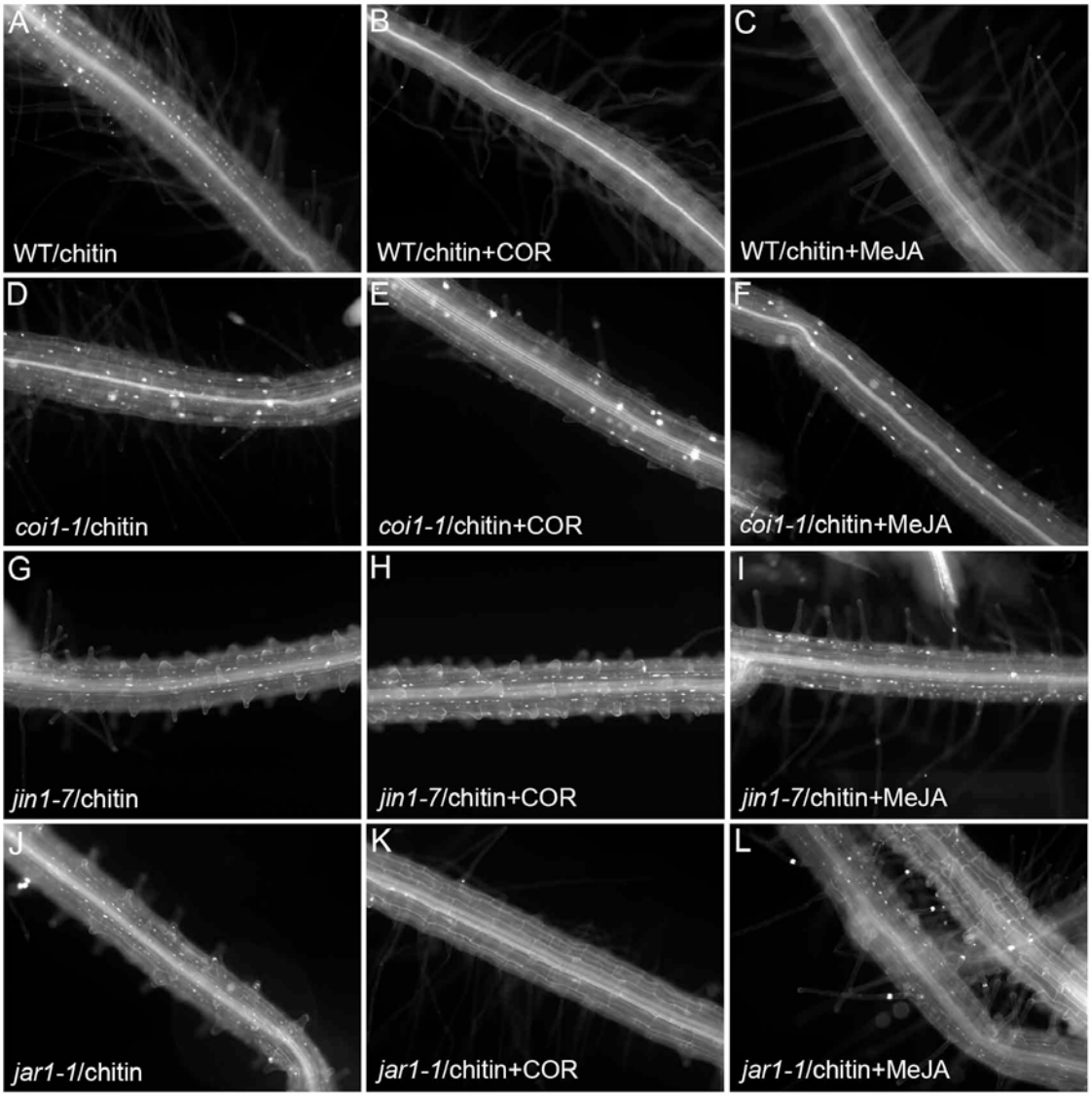

Figure S11. Suppression of the chitin-elicited callose deposition in the roots by COR and MeJA in Col-0, *coi1-1*, *jin1-7* and *jar1-1* seedlings.
Callose staining in the roots of Col-0 **(A, B, C)**; *coi1-1* **(D, E, F)**; *jin1-7* **(G, H, I)**; or *jar1-1* **(J, K, L)** seedlings treated with 500μg/mL chitin for 18h **(A, D, G, J)**; pre-treated with 5μM COR for 6h and treated with 500μg/mL chitin for 18h **(B, E, H, K)**; or pre-treated with 50μM MeJA for 6h and treated with 500μg/mL chitin for 18h **(C, F, I, L)**.

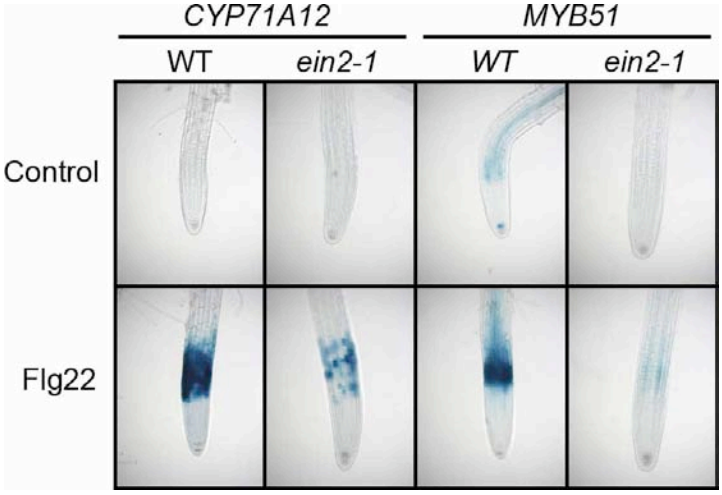

Figure S12. Overnight GUS staining in the roots of *CYP71A12*$_{pro}$*:GUS* and *MYB51*$_{pro}$*:GUS promoter:GUS* reporters in wild-type or *ein2-1* backgrounds.
Transgenic seedlings carrying *CYP71A12*$_{pro}$*:GUS*, *MYB51*$_{pro}$*:GUS* reporters in wild-type or *ein2-1* mutant backgrounds were treated with 100nM Flg22 for 3h (for *MYB51*) or 5h (for *CYP71A12*) before GUS staining. In this case the GUS staining was performed overnight instead of the normal 4h.

Chapter 4

A forward genetic screen to isolate new coronatine insensitive mutants

I. Introduction

Despite the fact that COR is the best-studied phytotoxin involved in plant pathogenesis, only two classes of mutants impaired in their response to COR have been identified in *Arabidopsis*, the *coi1* and *jin1* mutants (Feys et al., 1994; Laurie-Berry et al., 2006). COI1 is a major component of JA signaling and mediates the ubiquitination of JAZ proteins, negative regulators of the JIN1/MYC2 transcription factor. In presence of JA or COR, JAZ proteins are ubiquitinated and subsequently degraded by the proteasome, releasing MYC2, involved in many JA responses. Consistent with previously published reports, I showed that the *coi1-1* and *jin1-7* mutants are compromised for the COR-mediated suppression of the MAMP response in roots.

COR and JA both inhibit root growth in *Arabidopsis* (Feys et al., 1994; Staswick et al., 1992). This property was used in forward genetic screens with great success to isolate COR and JA insensitive mutants including *coi1*, *jar1* and *jin1* mutants (Feys et al., 1994; Lorenzo et al., 2004; Staswick et al., 1992). (*jar1* mutants were only isolated when JA was used as the selective agent since COR functions downstream of JAR1, an amino acid ligase that conjugates amino acids such as Ile to JA). However, screens for JA or COR resistant mutants using the root growth inhibition assay lacked sensitivity and all the mutants identified for resistance to COR were alleles of *COI1* (Feys et al., 1994).

In order to identify novel components of the JA/COR signaling pathway, I took advantage of the ability of COR to suppress the Flg22 response in roots to isolate new mutants impaired in their response to COR. The development of the liquid seedling assay and the generation of *promoter:GUS* reporter lines activated by MAMPs in the

roots gave me the tools to perform a much more sensitive forward genetic screen in an attempt to identify other players than COI1 and MYC2 involved in the COR-mediated suppression of the response to MAMPs. Specifically, I used transgenic plants expressing *CYP71A12*$_{pro}$*:GUS* to screen for mutants that expressed GUS in roots when treated with both Flg22 and COR.

II. Materials and Methods

II.1 EMS mutagenesis

~50,000 M1 transgenic *CYP71A12$_{pro}$:GUS* seeds were placed in a 50mL propylene conical tube. 40mL of water containing 0.3% EMS (ethyl methane sulfate) was added to the seeds and the tube was rotated for 15 hours. The seeds were washed 8 to 10 times with water and suspended in 0.1% agarose. ~5000 M1 seeds were sowed on 2ft^2 flats at a density of ~500 seeds per flat. M2 seeds were collected in 373 pools (~10 seeds/M1 plants from 10 M1 plants). EMS is extremely toxic and was handled with particular care (lab coat, double gloves, work in the fume hood). EMS was inactivated with 1M NaOH.

II.2 Growth of M2 plants

9-day old mutagenized *CYP71A12$_{pro}$:GUS* M2 seedlings were grown in 6-well microtiter dishes sealed with parafilm, each well containing ~40 seedlings and 2.5mL liquid Seedling Growth Medium (SGM; 1X MS basal medium with vitamins (Phytotechnology Laboratories) containing 0.5g/L MES hydrate, and 0.5% sucrose at pH5.7). ~80 M2 seedlings were grown from each pool of M1 plants (2 wells).

II.3 Treatment of M2 seedlings with Flg22 and COR

The seedlings were co-treated with 100nM Flg22 and 0.5μM COR for 5h before non-lethal GUS staining

II.4 Non-lethal GUS staining

After treatment with Flg22 and COR, seedlings were incubated for 1h at 37°C in 2.5mL of sterile non-lethal GUS substrate solution (50mM Sodium Phosphate pH7, 5mM EDTA, 0.5mM X-Gluc). Seedlings with GUS staining in their root tips were detected using magnifying glasses and transferred to Petri dishes containing 25mL of SGM medium solidified with 1% phytagar (PlantMedia) for recovery.

II. 5 Sequencing of *coi1* mutations

The genomic DNA of suspected *coi1* mutants were extracted as follows: a young leaf was placed in a 1.5mL microcentrifuge tube and ground in 400µL of extraction buffer (200mM Tris HCl pH 7.5, 250mM NaCl, 25mM EDTA, 0.5% SDS). The tube was centrifuged 5 minutes at 14,000 rpm and 300µL of the supernatant transferred to a new tube. DNA was precipitated by adding 300µL of isopropanol. After 2 minutes at room temperature, the tube was centrifuged 5 minutes at 14,000 rpm. The supernatant was removed and the pellet washed with 70% cold ethanol and let to dry on the bench. DNA was resuspended in 50µL of water.

The full length COI1 gene was amplified by PCR and sent for sequencing. The primers used for PCR and/or sequencing were as follows:

COI1-F1, TTG ATT CCA TCG TCC CAC TT

COI1-F2, GGC TGC TGC TGT TCT TCA TA

COI1-F3, CAT ACT TGG GAA TGC GTC CT

COI1-R1, AAG ACA ACA GAC AGT TGC ATG A

COI1-R2, ACA CAG TTT GTG GAA ACC CC

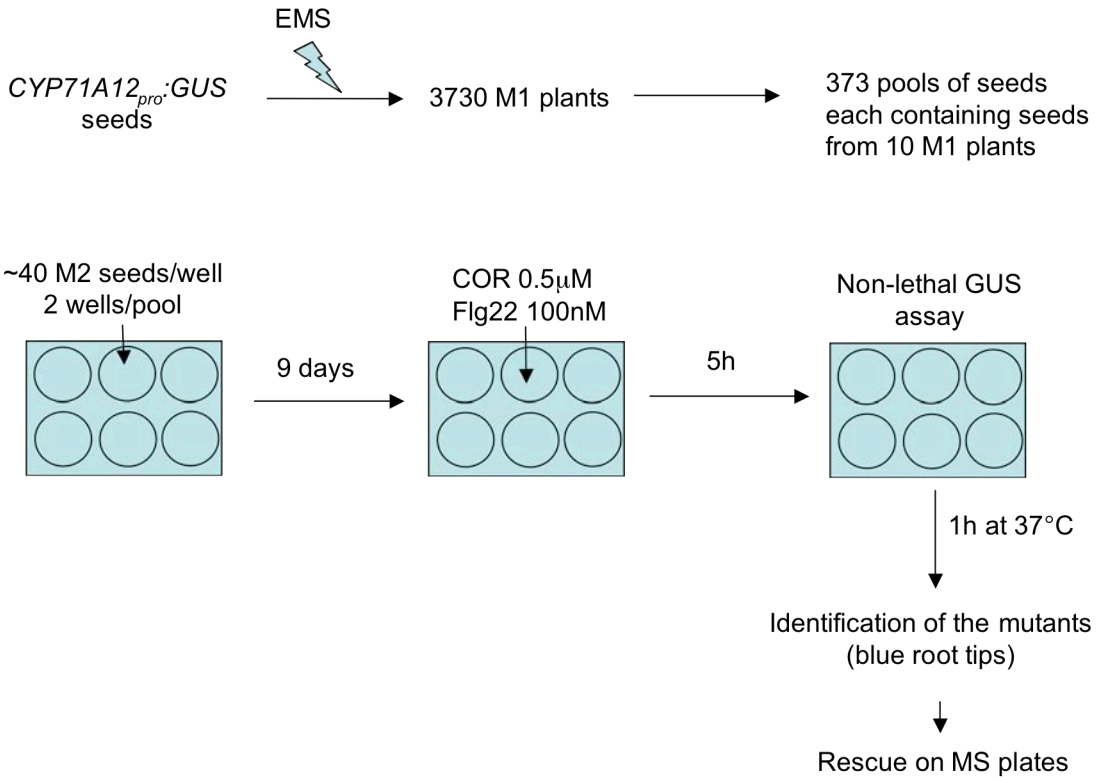

Figure 1. EMS screen to find mutants impaired for the COR-mediated suppression of the MAMP response in roots.

III. Results and Discussion

EMS mutagenized *CYP71A12$_{pro}$:GUS* seeds were screened for mutants that expressed the *CYP71A12$_{pro}$:GUS* reporter in response to Flg22 in the presence of COR. This work was performed with the help of high school student Diana Bartenstein. The screening strategy is outlined in Figure 1 and the results to date are summarized in Figure 2. Altogether, 90 mutants with confirmed phenotypes were identified in 290 independent pools of M2 seeds, 44 of which showed a strong phenotype with a complete lack of suppression of *CYP71A12$_{pro}$:GUS* by COR. To date, 26 mutants with strong phenotypes and 33 with leaky phenotypes have been backcrossed once with Col-0 as well as crossed to the Landsberg ecotype (Ler) for future mapping. Among the 90 mutants, 12 were male sterile, a well-known phenotype of *coi1* mutants (Feys et al., 1994). The CO1 locus in these 12 mutants was sequenced from PCR products generated using the primers shown in Figure 3. Eight of the 12 sequenced mutants had mutations in the sequenced region of the *COI1* gene (Figure 3), confirming the validity of the screen.

So far, 19 mutants have been tested for MeJA root growth inhibition. Of these, 11 had normal root growth inhibition, showing that at least 11 mutants most likely do not have a *coi1* or *jin1* mutations that confer total or partial MeJA insensitivity. This result suggests that other players than COI1 and JIN1/MYC2 involved in the COR-mediated suppression of the MAMP response in roots will most likely be identified from the screen. In comparison, a forward genetic screen performed by Feys et al. in 1994 for resistance to COR-mediated root growth inhibition only identified *coi1* mutants.

We expect the following classes of mutants from the screen: mutants upstream,

Figure 2. Current progress of the screen.

downstream or parallel to MYC2 and mutants conferring enhanced Flg22 response making it harder for COR to suppress. We are planning on categorizing the mutants by crossing them to a *35S:MYC2* transgenic line and to the *jin1-7* mutant. Mutants containing lesions upstream of MYC2 should be rescued by constitutive expression of MYC2 whereas mutants downstream of MYC2 should not. Mutants containing lesions in components parallel to (redundant with) MYC2 (which we would predict would have leaky phenotypes) should exhibit an increased lack of suppression (strong phenotypes) in the *jin1-7* background. Finally, mutants with an enhanced Flg22 response can easily be tested by GUS staining or qRT-PCR of Flg22-induced genes. We expect a higher level of Flg22-mediated activation of $CYP71A12_{pro}$*:GUS* in the absence of COR. Finally, the mapping of some of those mutants is underway and will help us to identify new players in COR and JA signaling as well as the Flg22 response in roots.

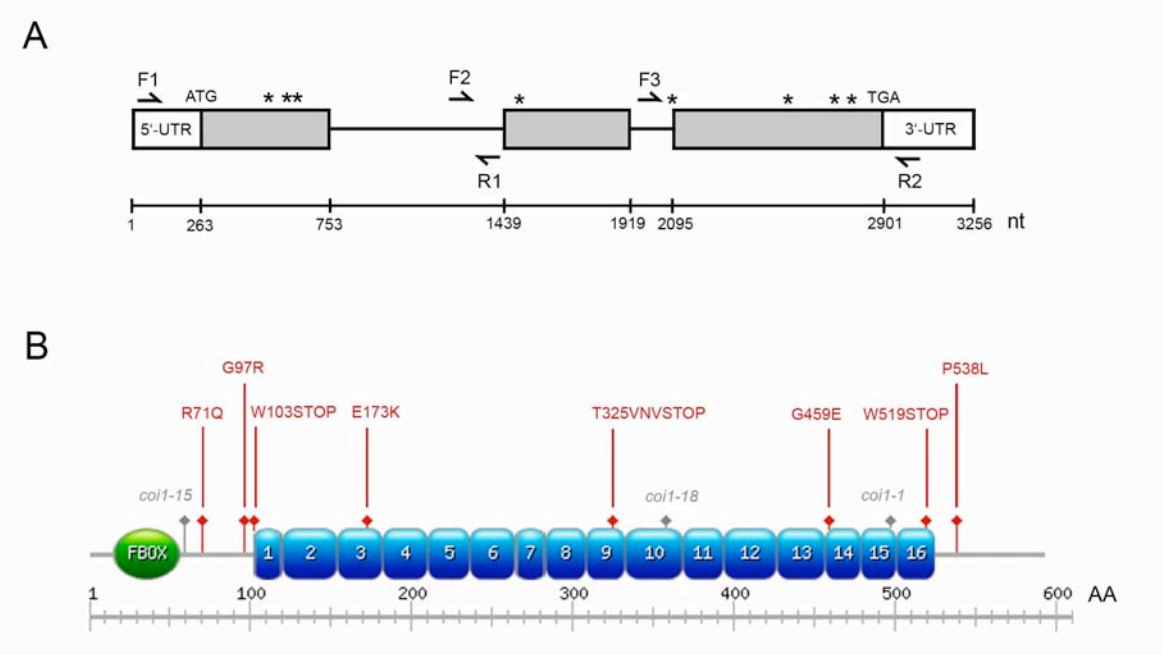

Figure 3. *coi1* mutations identified in the forward genetic screen.
(A) Schematic representation of the *COI1* gene indicating the sites of the *coi1* mutations found in the forward genetic screen (asterisks). The arrows indicate the primers used for sequencing.
(B) Schematic representation of the COI1 protein indicating in red the *coi1* mutations found in the screen and in gray, the previously characterized *coi1* mutations. The F-BOX domain is represented in green and the LRR domains in blue.

Chapter 5

Functional studies of the cytochrome P450

CYP76C2

I. Introduction

The cytochromes P450 are the largest family of plant enzyme proteins and are found in virtually all organisms from bacteria to humans. The number of P450s is particularly high in plants. 246 genes coding for P450s have been identified in *Arabidopsis thaliana* (plus 26 pseudogenes) and 356 in rice (*Oryza sativa*) (plus 99 pseudogenes). In comparison, the human genome "only" codes for 58 P450s (and 57 pseudogenes) (http://drnelson.utmem.edu/CytochromeP450.html). The vast number of P450s in plants is believed to be related to the emergence of a rich secondary metabolism. Over the course of evolution, plants developed an extremely various and complex set of chemicals, enabling them to grow in an aerial and competitive environment, to respond to biotic and abiotic stresses, and to communicate with other plants.

Cytochromes P450 are heme-binding proteins classified as monooxygenases. They use NADPH or NADH as electron donors and molecular O_2 as an oxygen donor. The reaction most often catalyzed by P450s is the insertion of one oxygen atom into a substrate that results in a hydroxylation as follows:

$$RH + O_2 + NADPH,H^+ \rightarrow ROH + H_2O + NADP^+$$

They can also catalyze a variety of other reactions involving oxygenation, including epoxidations and nitrogen and sulfur oxidations. P450s also catalyze dealkylations, isomerizations, dimerizations, dehydrations, carbon-carbon cleavage, decarboxylations, dehalogenations and deaminations (Sono et al., 1996). The diverse

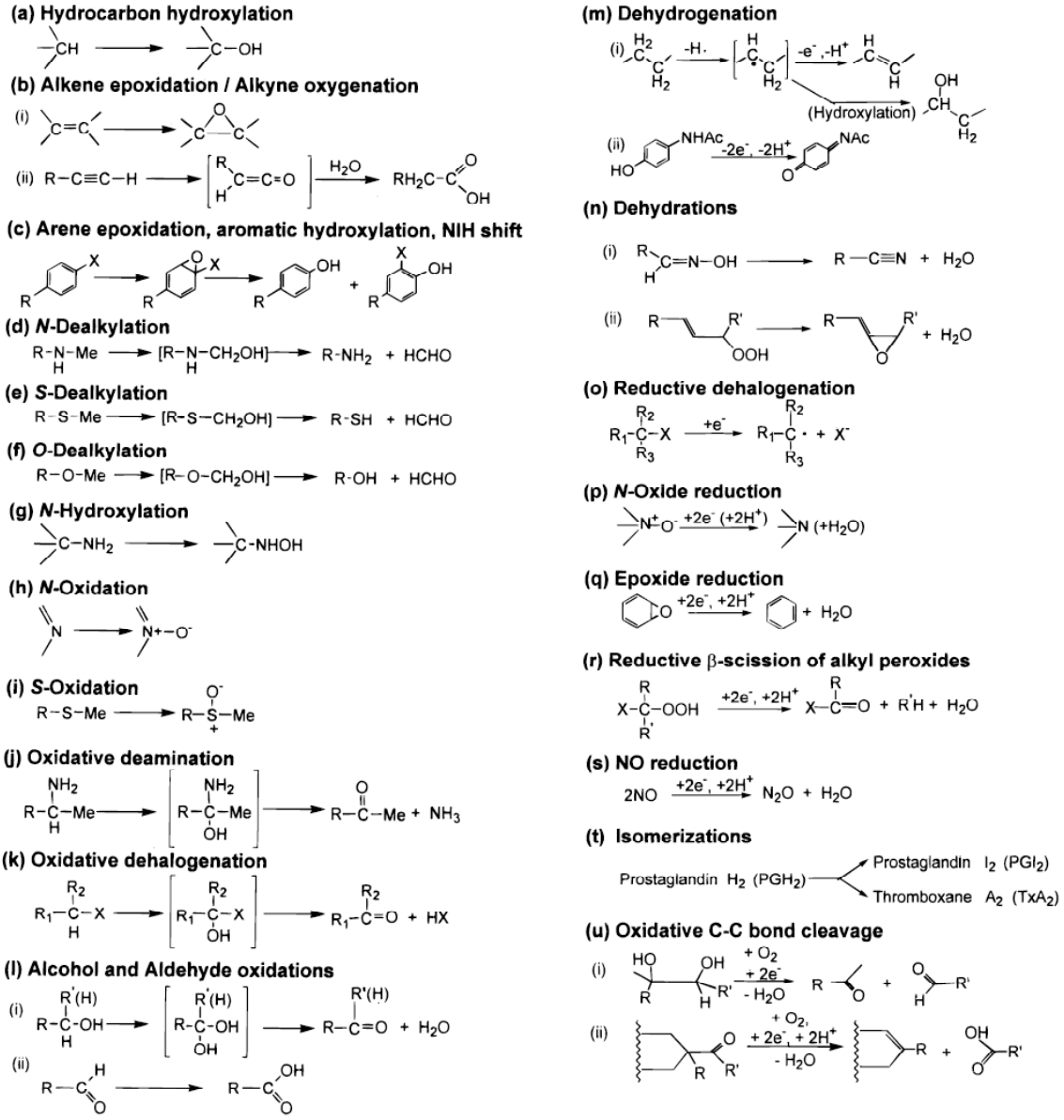

Figure 1. Diverse reactions catalyzed by cytochromes P450.
Figure from Sono et al., 1996.

reactions catalyzed by P450s are summarized in Figure 1. P450s can also bind to carbon monoxide in their reduced state instead of O_2. This shifts the maximum of absorbance of the heme to 450 nm. The family took its name from this property (**P**igment absorbing at **450** nm).

So far, all the P450s described in plants are membrane-bound, most often on the cytosolic side of the endoreticulum. However, a number of *Arabidopsis thaliana* P450s are described or predicted to be targeted to the chloroplasts or mitochondria, based on their signal peptides (Schuler, 2006; Werck-Reichhart, 2002). P450s are usually coupled with an NADPH-cytochrome reductase or NADH-cytochrome b_5 reductase and cytochrome b_5 (Werck-Reichhart, 2002). Those proteins allow the sequential transfer of electrons from NADPH or NADH to the heme and the reduction of Fe^{3+} to Fe^{2+} leading to O_2 activation. The catalytic cycle of a P450-mediated hydroxylation is shown in Figure 2 (Werck-Reichhart and Feyereisen, 2000).

The natural substrates of P450s are highly diverse. P450s have been shown to play a role in the biosynthesis of structural polymers (lignin, cutins, suberins), UV protectants (flavonoids, coumarins, sinapoyl esters), antioxidants, pigments (anthocyanins, carotenoids), hormones (auxin, jasmonate, brassinosteroids, gibberellins) and defense compounds (isoflavonoids, glucosinolates, terpenes etc.). In addition, P450s are known to detoxify exogenous molecules such as pesticides and pollutants, known as xenobiotics (Schuler and Werck-Reichhart, 2003).

A number of P450s have been shown to play important roles for plant defense against pathogens in *Arabidopsis thaliana*, in particular for the biosynthesis of the signaling molecule oxylipin jasmonate, the biosynthesis of antimicrobial compounds

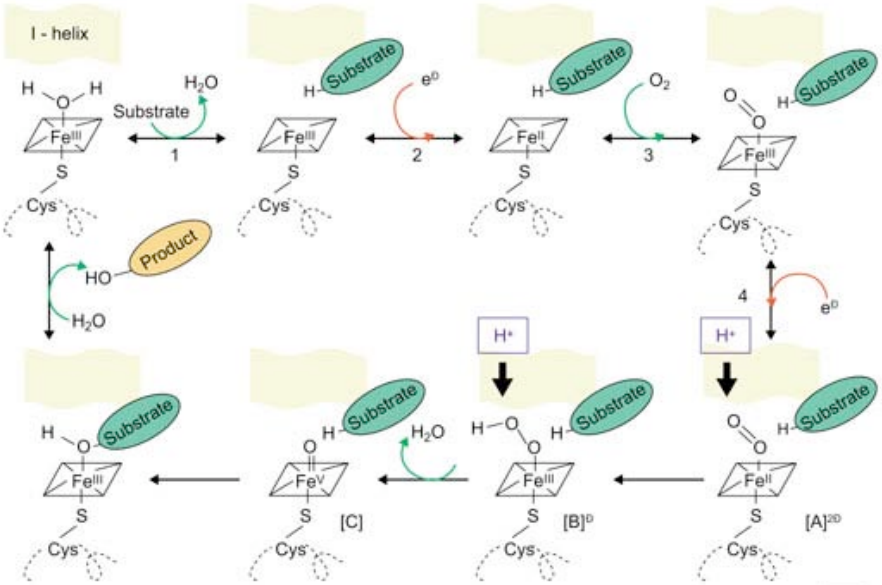

Figure 2. Catalytic cycle of cytochromes P450s**.**
P450s are classified as monooxygenases and they most often catalyze an hydroxylation. The reaction involves a one-electron reduction of the heme Fe^{3+} to Fe^{2+} leading to the binding of O_2 followed by a second one-electron reduction leading to the formation of the activated oxygen intermediate species $[A]^{2D}$ protonated to form the $[B]^{D}$ intermediate. $[B]^{D}$ is subsequently protonated to form water and the very reactive and electrophilic iron-oxo intermediate [C]. It is usually [C] that reacts with the substrate to form the hydroxylated product.
Figure from Werck-Reichhart and Feyereisen, 2000.

Gene	Accession #	Function	Pathway	References
CYP71A13	AT2G30770	conversion of indo-3-acetaldoxime (IAOx) to indole-3-acetonitrile (IAN)	camalexin	(Nafisi et al., 2007)
CYP71B15/PAD3	AT3G26830	conversion of dihydrocamalexic acid to camalexin	camalexin	(Schuhegger et al., 2006)
CYP74A1	AT5G42650	allene oxide synthase	jasmonate	(Laudert et al., 1996)
CYP79A2	AT5G05260	Conversion of L-Phenylalanine to Phenylacetaldoxime	benzylglucosinolate	(Wittstock and Halkier, 2000)
CYP79B2	AT4G39950	Conversion of Trp to indo-3-acetaldoxime (IAOx)	indole glucosinolates	(Hull et al., 2000; Mikkelsen et al., 2000)
CYP79B3	AT2G22330	Conversion of Trp to indo-3-acetaldoxime (IAOx)	indole glucosinolates	(Hull et al., 2000)
CYP79F1	AT1G16410	Conversion of homomethionine to Aldoximes	aliphatic glucosinolates	(Hansen et al., 2001)
CYP79F2	AT1G16400	Conversion of homomethionine to Aldoximes	aliphatic glucosinolates	(Chen et al., 2003)
CYP81F2	AT5G57220	4-hydroxylase for IGS to 4M-IGS	indole glucosinolates	(Bednarek et al., 2009; Clay et al., 2009)
CYP83A1/REF2	AT4G13770	conversion of aldoximes to thiohydroximates	glucosinolates	(Naur et al., 2003)
CYP83B1/ATR4/SUR2	AT4G31500	conversion of aldoximes to thiohydroximates	indole glucosinolates	(Naur et al., 2003)
CYP86A2	AT4G00360	fatty acid hydroxylase	Fatty acids. cuticle	(Xiao et al., 2004)

Table 1. Cytochromes P450 involved in plant defense.

such as indolic glucosinolates and the phytalexin camalexin, or in lipid metabolism for the development of the cuticle. Those P450s are summarized in Table 1. However, a number of P450s that are transcriptionally activated by pathogens have not been characterized. These are potentially important in plant defense, either for the synthesis of signaling molecules or defense compounds. Discovering the function of those P450s is an important part of trying to better understand plant defense responses. This chapter focuses on one of these P450s, CYP76C2.

Microarray data analysis performed at MGH in the Ausubel laboratory by Carine Denoux and Julia Dewdney revealed that a number of cytochromes P450 are highly induced by two MAMPs, Flg22 and OGs (Table 2) (Denoux et al., 2008). Among these P450s, some had already been shown to be involved in plant defense mechanisms, such as *CYP71B15* and *CYP71A13,* which are required for the biosynthesis of camalexin (Ferrari et al., 2003; Nafisi et al., 2007; Schuhegger et al., 2006; Thomma et al., 1999), or *CYP79B2* and *CYP79B3* for the biosynthesis of indole glucosinolates and camalexin (Bednarek et al., 2009; Clay et al., 2009; Kim et al., 2008). However, many of these p450s have not been characterized yet. Among the uncharacterized P450s, four are highly up-regulated by Flg22 or OGs: *CYP76C2*, *CYP81D8*, *CYP82C2* and *CYP82C3*. I decided to focus my attention on *CYP76C2* since it was previously described as activated during the hypersensitive response, a major response of plants to pathogens (Godiard et al., 1998). Moreover, a *promoter:GUS* transgenic reporter line and a 35S overexpressing line had already been generated for the *CYP76C2* gene by Sébastien Grec, a former post-doctoral fellow in Danièle Werck's laboratory.

Gene	Accession #	Flg22 1h	Flg22 3h	OGs 1h	OGs 3h	Putative or real function	Pathway	References
CYP71A12	AT2G30750	25.37	100.00	9.08	18.35	conversion of indo-3-acetaldoxime (IAOx) to indole-3-acetonitrile (IAN)	camalexin	
CYP71A13	AT2G30770	1.74	13.76	1.48	2.11	conversion of indo-3-acetaldoxime (IAOx) to indole-3-acetonitrile (IAN)	camalexin	(Nafisi et al., 2007)
CYP71A18	AT1G11610	1.70	6.22	1.01	-1.22			
CYP71B14	AT5G25180	2.46	3.95	2.57	1.36			
CYP71B15/PAD3	AT3G26830	5.83	16.91	3.88	11.47	conversion of dihydrocamalexic acid to camalexin	camalexin	(Schuhegger et al., 2006)
CYP71B2	AT1G13080	1.31	2.60	1.29	1.09			
CYP71B23	AT3G26210	2.95	4.62	3.07	1.73			
CYP71B24	AT3G26230	2.06	3.07	2.08	1.30			
CYP71B29	AT1G13100	1.44	3.27	1.01	1.14			
CYP71B5	AT3G53280	5.48	2.02	2.57	-1.17			
CYP71B6	AT2G24180	3.18	7.02	1.90	2.20			
CYP71B7	AT1G13110	5.12	7.64	2.98	1.45			
CYP72C1	AT1G17060	4.15	4.12	3.19	-1.56			
CYP73A5	AT2G30490	3.90	9.77	2.97	3.18	cinnamic acid 4-hydroxylase	Phenylpropanoids	(Mizutani et al., 1997)
CYP76C2	AT2G45570	2.05	11.59	-1.26	1.85			
CYP79B2	AT4G39950	6.47	29.53	4.56	5.51	Conversion of Trp to indo-3-acetaldoxime (IAOx)	indole glucosinolates	(Hull et al., 2000; Mikkelsen et al., 2000)
CYP79B3	AT2G22330	-1.12	3.50	-1.24	2.64	Conversion of Trp to indo-3-acetaldoxime (IAOx)	indole glucosinolates	(Hull et al., 2000)
CYP81D8	AT4G37370	26.17	6.25	10.47	-1.01			
CYP81F2	AT5G57220	100.00	100.00	100.00	62.81	4-hydroxylase for IGS to 4M-IGS	indole glucosinolates	(Bednarek et al., 2009; Clay et al., 2009)
CYP81G1	AT5G67310	6.37	3.89	5.88	2.70			
CYP82C2	AT4G31970	4.27	13.41	1.70	1.11			
CYP82C3	AT4G31950	100.00	26.60	42.70	2.71			
CYP83B1/ATR4/SUR2	AT4G31500	4.60	10.18	4.57	5.52	conversion of aldoximes to thiohydroximates	indole glucosinolates	(Naur et al., 2003)
CYP84A1	AT4G36220	1.32	3.40	1.79	4.75	5-hydroxylase for coniferaldehyd, coniferyl alcohol and ferulic acid	Phenylpropanoids	(Meyer et al., 1996)
CYP86A2	AT4G00360	1.52	2.35	1.27	1.56	fatty acid hydroxylase	Fatty acids, cuticle	(Xiao et al., 2004)
CYP94C1	AT2G27690	20.05	61.44	14.78	4.96	fatty acid omega hydroxylase	Fatty acids	
CYP98A3	AT2G40890	1.33	3.26	1.29	1.31	3'-hydroxylase of p-coumaryl shikimic/quinic acids	Phenylpropanoids	(Schoch et al., 2001)
CYP706A2	AT4G22710	5.00	2.66	4.08	1.61			
CYP707A3	AT5G45340	10.13	1.97	8.98	-1.28	ABA 8'-hydroxylase	ABA degradation	(Saito et al., 2004)
CYP710A1	AT2G34500	1.11	5.50	1.05	1.55	sterol C-22 desaturase	Sterols	(Morikawa et al., 2006)

Table 2. List of cytochromes P450 significantly induced by Flg22 and OGs in *Arabidopsis thaliana.*
Data represent the fold-change value between the treatment and the water control. Fold changes superior to 2 are indicated in yellow, fold changes superior to 10 are indicated in red. Data based on Denoux et al., Molecular Plant, 2008 (Denoux et al., 2008).

II. Materials and Methods

II.1. Plant growth conditions

For GUS staining, RT-PCR or infections in adult leaves, *Arabidopsis thaliana* plants were grown on 2 mix professional formula soil (Fafard) for 4-5 weeks under 12h of daylight (75μE), 18°C at night, 22°C during the day, and 60% humidity. In the case of GUS staining in senescing leaves, plants were grown until the first senescing leaves appeared. In the case of GUS staining in siliques, plants were grown until flowering and mature siliques were collected at different stage of senescence.

For GUS staining, or RT-PCR in *Arabidopsis thaliana* seedlings, plants were grown in 12-well microtiter dishes sealed with parafilm, each well containing 10 to 15 plants and 1mL of seedling growth medium (SGM; 1X MS basal medium with vitamins (Phytotechnology Laboratories), 0.5g/L MES hydrate, 0.5% sucrose, pH5.7). Seedlings were grown for 10 days at 22°C in a plant growth chamber under 16 hours of light (100μE). The medium was changed on day 8.

For plants grown on horizontal agar plates, seedlings were grown on SGM medium solidified with 1% phytagar (PlantMedia) at 22°C in a plant growth chamber under 12 hours of daylight (100μE).

For plants grown on vertical agar plates, seedlings were grown on 20x100mm Petri dishes containing SGM solidified with 1% phytagar (PlantMedia) at 22°C in a plant growth chamber under 12 hours of daylight (75 μE).

II.2. Bacterial infection

Pseudomonas syringae bacterial strains were cultured on King's Broth (KB) plates supplemented with appropriate antibiotics: 50μg/mL rifampycin for *P. syringae pv. tomato (Pst)* DC3000 and *Pst*DC3000 *hrcC*, 50μg/mL kanamycin for *PstDC3000/AvrRPT2*. Bacteria were grown overnight in KB supplemented with an appropriate antibiotic at 28°C. Bacteria were centrifuged, washed three times with water, and resuspended in water to the appropriate final OD_{600}.

For GUS staining or RT-PCR in adult leaves, the lower surfaces of leaves were infiltrated with a bacterial suspension of OD_{600} 0.002 using a 1mL syringe. For bacterial counts in adult leaves, leaves were infiltrated with a bacterial suspension of OD_{600} 0.0002. In the case of 10 day-old seedlings, plants were infected by adding 50μL of bacterial suspension into each well to a final OD_{600} of 0.0002.

II.3. *Botrytis cinerea* infections

B. cinerea was cultivated on plates containing MEP media (1% proteose peptone, 2% malt extract, 2% glucose, 1.5% agar) sealed with 3M surgical tape (Micropore) and placed under 12h of daylight (75 μM), 18°C at night, 22°C during the day, 80% humidity, until sporulation (~2 weeks). Spores were harvested by flooding the plate with sterile water, scraping the surface with a glass rod, collecting and filtering the suspension through a sterile gauze pad. The spore suspension was then centrifuged at 3000 rpm for 10 minutes and resuspended in PDB (Potato dextrose broth). The spore concentration was determined using a hemocytometer and adjusted to 5.10^5/mL in PDB. Leaves were infected by placing a 5μL drop of spore suspension on each side of the mid-vein and the plants were covered with a plastic dome.

II.4. GUS histochemical assay

The transcriptional activation *CYP76C2* was studied using a *promoter:GUS* transgenic line generated by Sébastien Grec, a former post-doctoral fellow in Danièle Werck-Reichhart's laboratory in Strasbourg. The GUS histochemical assay was performed as followed. After treatment with bacteria and/or elicitors, etc., plants were washed with 50mM Sodium Phosphate buffer pH7 and incubated in GUS substrate solution (50mM Sodium Phosphate pH7, 10mM EDTA, 0.5mM $K_4[Fe(CN)_6]$, 0.5mM $K_3[Fe(CN)_6]$, 0.5mM X-Gluc, 0.01% Silwet L-77). The tissues were vacuum-infiltrated for 5min for seedlings, 20 minutes for adult leaves, and then incubated at 37°C for 6 to 8 hours. Tissues were then fixed with a 3:1 ethanol:acetic acid solution at 4°C overnight and placed in ethanol 95%.

II.5. Nitroblue tetrazolium (NBT) staining in seedling roots

After treatments, seedlings were placed in a 2mM NBT, 20mM phosphate buffer pH6.1 for 15 minutes. The reaction was stopped by placing the seedlings in water.

II.6. Bacterial count in adult infected leaves

For cfu (colony forming units) experiments, 4 leaves of 6 different plants were infiltrated with bacteria at an initial OD_{600} of 0.0002. After 3 days, 6mm disks in the infected area were collected from each infected leaves (2 disks per leaves) and ground in 100µL of water. The number of colony forming units (cfus) per cm^2 was determined by 10X serial dilution of the initial grinding solution. A 5µL drop of each dilution was

plated on LB plates supplemented with the proper antibiotic and the number of bacterial colonies for each dilution counted.

II.7. Treatment with elicitors, hormones, toxins, herbicides

Elicitors, hormones, toxins, were used at the following concentrations unless otherwise specified: 1μM Flg22, 10μM SA, 10μM ABA, 25mM H_2O_2, 1μM fumonisin B1 (FB1), 1μM isoproturon, 10nM paraquat. All stock solutions were prepared in water except the ABA stock solution, which was prepared at 50mM in 50% ethanol. For treatments in adult plants, Flg22 was simply infiltrated in the leaves using a 1mL syringe as for bacterial infiltration. For treatments in seedlings, the elicitor, hormone or toxin was simply added directly to the liquid growth media. As a control, an equal amount of solvent was added in the medium or infiltrated in the leaf.

II.8. RNA extraction and RT-PCR analysis

For RT-PCR in seedlings, total RNA was extracted from approximately 15 seedlings. In the case of RT-PCR in adult infected leaves, total RNA was extracted from 3 infected leaves (same stage) coming from 3 different plants. RNA was extracted using the RNeasy plant mini kit (Qiagen) according to the manufacturer's instructions. The leaves were snap frozen by liquid nitrogen and ground using a mortar and pestle. Total RNA was treated with DNAse I (Ambion) to avoid genomic DNA contamination and 1μg of total RNA was reverse-transcribed using the iScript cDNA synthesis kit from Biorad. RT-PCR was performed using a DNA engine tetrad 2 PCR machine (Peltier) and Taq polymerase (Roche). The program used for RT-PCR was as follows: 3 minutes at

94°C, and n cycles of 30 seconds at 94°C, 30 seconds at 55°C and 40 seconds at 70°C. The primers used for RT-PCR were:

CYP76C2-F, TCA TCG GAA ACA TTC ACC TTG

CYP76C2-R, GAT CAA GGA ACC CCA GAA ATG

PR1-F, CCA CAA GAT TAT CTA AGG GT

PR1-R, TAG TTG TTC TGC GTA GCT C

UBQ5-F, CCT AAC GGG GAA GAC CAT AAC

UBQ5-R, AGG ATC GAT CTA CCG CTA CAA

II.9. Mutant seed stocks

Insertion mutants used in this work (obtained from the Arabidopsis Biological Resource Center, Columbus, HO): *cyp76C2* (SALK_037019), *cyp76C4-1* (SALK_093179), *cyp76C4-2* (SALK_006831), *cyp76C4-3* (SALK_071239). The primers used to genotype the lines and isolate homozygotes were as follows:

037019_LP, TCATCGGAAACATTCACCTTG

037019_RP, GATCAAGGAACCCCAGAAATG

093179_LP, TCCTCGGTTTAGGCTAGGAAG

093179_RP, GAAATTAGGTTTGTACCTCCAACG

006831_LP, GATGATTTTGCTTAGTCATTTGAG

006831_RP, CATAGCTACCGAGATCAACCG

071239_LP, GCAACACATAAAAGTCAATTAACAC

071239_RP, GATGTGGAGGTTCTTGGGTTC

LBb1, GCGTGGACCGCTTGCTGCAACT

The *fls2* mutant (SAIL_691_C04) was provided by Jeffrey Dangl (University of North Carolina at Chapel Hill).

III. Results

III.1 *CYP76C2* expression analysis

III.1.1. Expression in response to pathogens and elicitors

The expression of *CYP76C2* after various treatments was studied using a *CYP76C2$_{pro}$:GUS* transgenic line generated by Sébastien Grec. The GUS reporter was highly induced by *P. syringae pv. tomato* DC3000 (*Pst*DC3000) in adult leaves 12h and 24h after infection (Figure 3). The activation was restricted to the zone of infiltration and no staining was observed in systemic tissues showing that *CYP76C2* is expressed locally after infection. *CYP76C2$_{pro}$:GUS* was also activated after infection with the avirulent strain *Pst*DC3000*/AvrRPT2,* which induces an HR. In the latter case, stronger staining was observed after infection (Figure 3). The observation that *CYP76C2* responds more strongly during an avirulent interaction triggering HR was previously described (Godiard et al., 1998). Interestingly, *CYP76C2$_{pro}$:GUS* was also induced after inoculation by a *hrcC* mutant of *Pst*DC3000, impaired for the injection of its type three effectors into the plant cells, and thus unable to infect plant tissue or produce any symptoms. However, after 24h, I observed a decrease in staining elicited by *Pst*DC3000 *hrcC* compared with *Pst*DC3000 and *Pst/AvrRPT2*, probably due to the fact that the *hrcC* mutant bacteria are unable to grow *in planta*. No staining was detected after infiltration of a water control suggesting that *CYP76C2* does not simply respond to tissue damage resulting from the infection process. The *CYP76C2$_{pro}$:GUS* reporter also responded to infiltration of leaves with Flg22, consistent with what was found by microarray in seedlings (Denoux et al.,

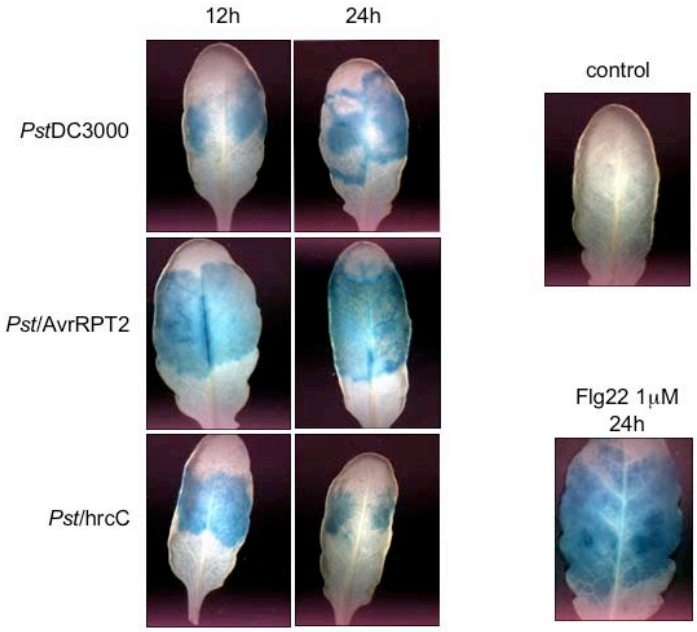

Figure 3. *CYP76C2* is induced by *P. syringae* infection and Flg22 treatment in adult leaves.
GUS staining of 4-week old $CYP76C2_{pro}$*:GUS* leaves infiltrated with bacteria at an initial OD_{600} of 0.002 or infiltrated with Flg22 1μM. Control was infiltrated with water.

2008). Those results suggest that *CYP76C2* could be activated by *Pst*DC3000-encoded MAMPs and that *Pst*DC3000 type III effectors do not suppress that activation.

The GUS results were confirmed by RT-PCR in seedlings infected by *P. syringae* in the 12 well plate format. *CYP76C2* was strongly induced by *Pst*DC3000 at 12h and 24h post-inoculation (Figure 4). Consistent with the GUS results obtained in adult leaves, this activation was stronger in seedlings infected with *Pst/AvrRPT2*. *CYP76C2* expression correlated well with *PR1* induction, a marker of HR and SA signaling. Similar results were obtained after infection with the two *P. syringae pv. maculicola* strains *Psm*ES4326 and *Psm/AvrRPT2*. Some increase in *CYP76C2* expression was also detected after infiltration with *Pst/hrcC* but this activation did not increase over time, consistent with the GUS results and this strain's incapacity to cause a successful infection.

As previously discussed in the general introduction to this thesis, HR is known to trigger the accumulation of SA, a major plant defense hormone. In particular, SA is essential for the activation of systemic acquired resistance (SAR) and mutants impaired in SA production like *sid2,* or SA signaling like *npr1,* are more sensitive to *P. syringae* both locally and systemically (Cao et al., 1994; Wildermuth et al., 2001). Since *CYP76C2* expression is increased during HR, I tested to see if *CYP76C2* was an SA-responsive gene. Indeed, SA induced *CYP76C2* as early as 3h after treatment in seedlings (Figure 4). The efficiency of the SA treatment was confirmed by the activation of the SA marker *PR1*. In addition to HR, Flg22 treatment is also known to trigger the activation of the SA pathway and SAR (Mishina and Zeier, 2007; Tsuda et al., 2008). Consistently, *CYP76C2* and, to a lower extent *PR1,* were induced by Flg22 at 3h after

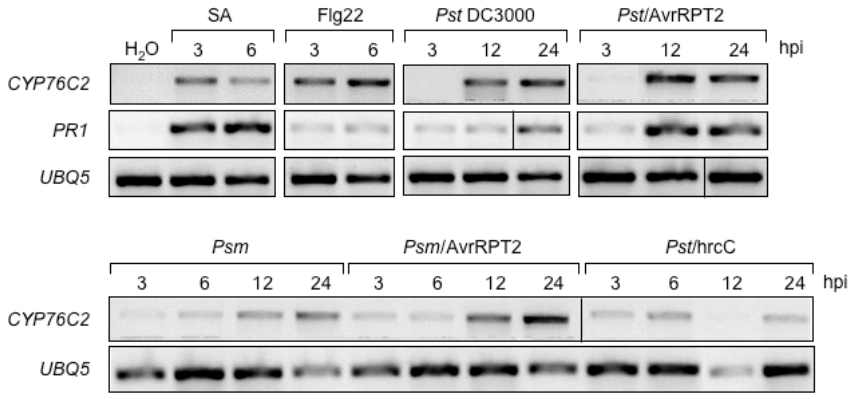

Figure 4. *CYP76C2* is activated by SA, Flg22 and *P. syringae* in seedlings.
RT-PCR in 10 day-old *Arabidopsis* seedlings treated with 10μM SA, 1μM Flg22 or infected with different strains of *P. syringae* at an initial OD_{600} of 0.0002. Ubiquitin 5 (UBQ5) was used as a control. hpi: hours post inoculation.

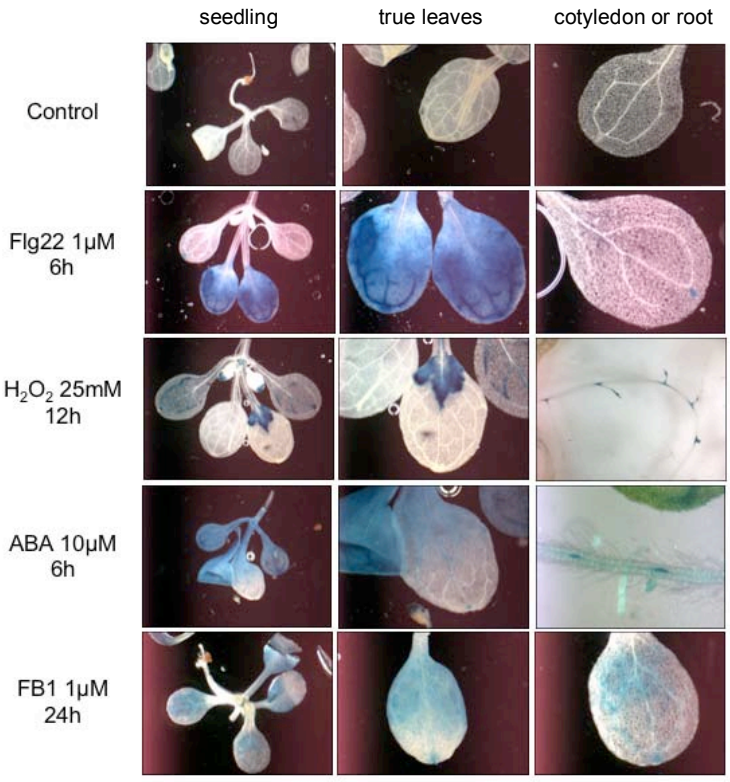

Figure 5. *CYP76C2* is induced by Flg22, H_2O_2, ABA and fumonisin B1 in seedlings. GUS staining in 10 day-old *CYP76C2*$_{pro}$*:GUS* seedlings treated with 1µM Flg22, 25mM H_2O_2, 10µM ABA and 1µM fumonisin B1 (FB1).

treatment in seedlings. This was confirmed by looking at *CYP76C2*$_{pro}$*:GUS* activation in seedlings after Flg22 treatment for 3h (Figure 5). Interestingly, *CYP76C2* was only activated in the seedling's true leaves but not in the roots or the cotyledons with the exception of hydathodes. This is not due to a lack of Flg22 sensitivity in the roots or in the cotyledons since those organs were both shown to respond to Flg22 (Clay et al., 2009) (Chapter 2). It was shown that in *Arabidopsis* seedlings, cotyledons are much more sensitive to downy mildew (*Hyaloperonospora parasitica*) than the first set of true leaves (McDowell et al., 2005). This increased resistance in true leaves is dependent on the SA defense pathway. Indeed the SA mutants *pad4* and *npr1* as well as the transgenic line *nahG* unable to accumulate SA are greatly impaired for this resistance in true leaves. Therefore, it is possible that cotyledons lack some components of the SA pathway explaining why the SA-responsive gene *CYP76C2* is not expressed in cotyledons after Flg22 treatment.

CYP76C2$_{pro}$*:GUS* adult plants were also infected with the necrotrophic pathogen *Botrytis cinerea* known to trigger the accumulation of SA locally (Veronese et al., 2006). *CYP76C2* was activated in a narrow band directly surrounding the lesion (Figure 6). This localization is similar to what was found for the *PR1:GUS* reporter line (Ferrari et al., 2003). Altogether, these data suggest that *CYP76C2* is activated by SA in response to MAMPs or pathogens. Additional evidence that *CYP76C2* is regulated by SA signaling is presented below.

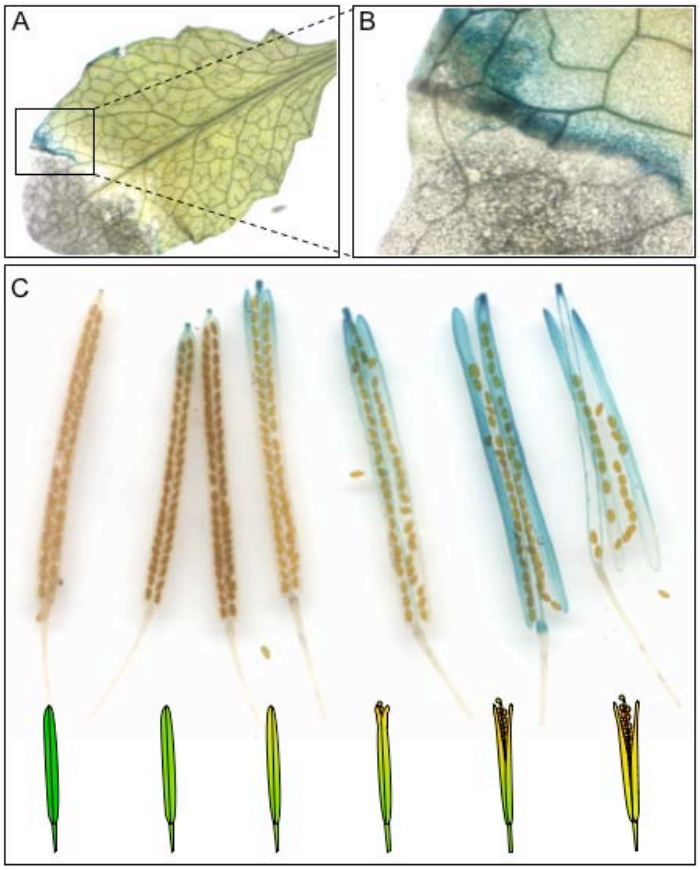

Figure 7. CYP76C2 is activated during senescence.
GUS staining in *CYP76C2*$_{pro}$*:GUS* senescing leaves **(A, B)** and opening siliques **(C)**.

III.1.2. Expression in senescing tissues

In addition to being responsive to *P. syringae*, *CYP76C2* has been shown to be activated during developmental cell death (Godiard et al., 1998). The activity of the *CYP76C2*$_{pro}$*:GUS* reporter was monitored during plant development, in particular during senescence. The reporter was activated in senescing leaves at the junction between dead and alive tissue (Figure 7A; 7B), reminiscent of what was found with *B. cinerea* infection. The reporter was also expressed in dehiscing siliques (Figure 7C), known to undergo an active senescence program to allow the seeds to be relieved (Wagstaff et al., 2009).

SA is known to play a role in promoting leaves senescence. Indeed, it has been shown that SA accumulates in senescing tissues. Moreover, transgenic plants carrying the SA-degrading enzyme gene *nahG,* and the SA mutants *pad4* and *npr1* show retarded senescence (Morris et al., 2000). *CYP76C2* may be activated by SA during senescence, but no experiments were done in senescing leaves of SA mutants to confirm this hypothesis.

The plant hormone abscisic acid (ABA) is known to trigger senescence (Zeevaart and Creelman, 1988) and *CYP76C2* has been shown to respond to ABA treatment (Godiard et al., 1998). Consistent with this result, the *CYP76C2* GUS reporter was highly induced by ABA in the seedling assay, in all the part of the plant (Figure 5), including the roots and the cotyledons unlike what was found for Flg22. It is possible that ABA-triggered senescence is independent of SA or acting downstream of SA production.

III.1.3. Expression related to oxidative stress

During HR or senescence, ROS production is believed to play a major role in triggering cell death and inducing the production of SA (Alvarez et al., 1998; Woo et al., 2004). H_2O_2, an important ROS, induced the *CYP76C2*$_{pro}$*:GUS* reporter in seedlings in both roots and shoots (Figure 5). In true leaves, *CYP76C2* expression was localized at the junction between damaged tissue starting to bleach and tissue showing no sign of bleaching, similar to the staining observed in senescing leaves. In roots, *CYP76C2* expression was localized at the junction between the main root and lateral roots and at the root tip, corresponding, perhaps, to regions of greater permeability. In addition, fumonisin B1 (FB1), a mycotoxin produced by the maize pathogen *Fusarium moniliforme* that is known to trigger oxidative stress and cell death (Stone et al., 2000), also activated the *CYP76C2*$_{pro}$*:GUS* reporter (Figure 5). This activation was localized in the cotyledons and the true leaves but absent in the roots.

The absence of FB1-elicited expression in roots compared to what was observed in the case of H_2O_2 and ABA treatments, could be due to the absence of photosynthesis in the roots. Indeed, induction of cell death by FB1 is known to require light and could involve ROS produced during photosynthesis. FB1 may inhibit ROS-scavenging enzymes such as ascorbate peroxidase and catalase during photosynthesis leading to an oxidative stress and cell death (Stone et al., 2000). Flg22 have been shown to trigger an oxidative burst and SA accumulation in leaves, but no evidence has shown so far that this occurs in roots. It is possible that Flg22 does not activate *CYP76C2* in the roots because no oxidative burst or SA accumulation occurs after Flg22 treatment. To try to address

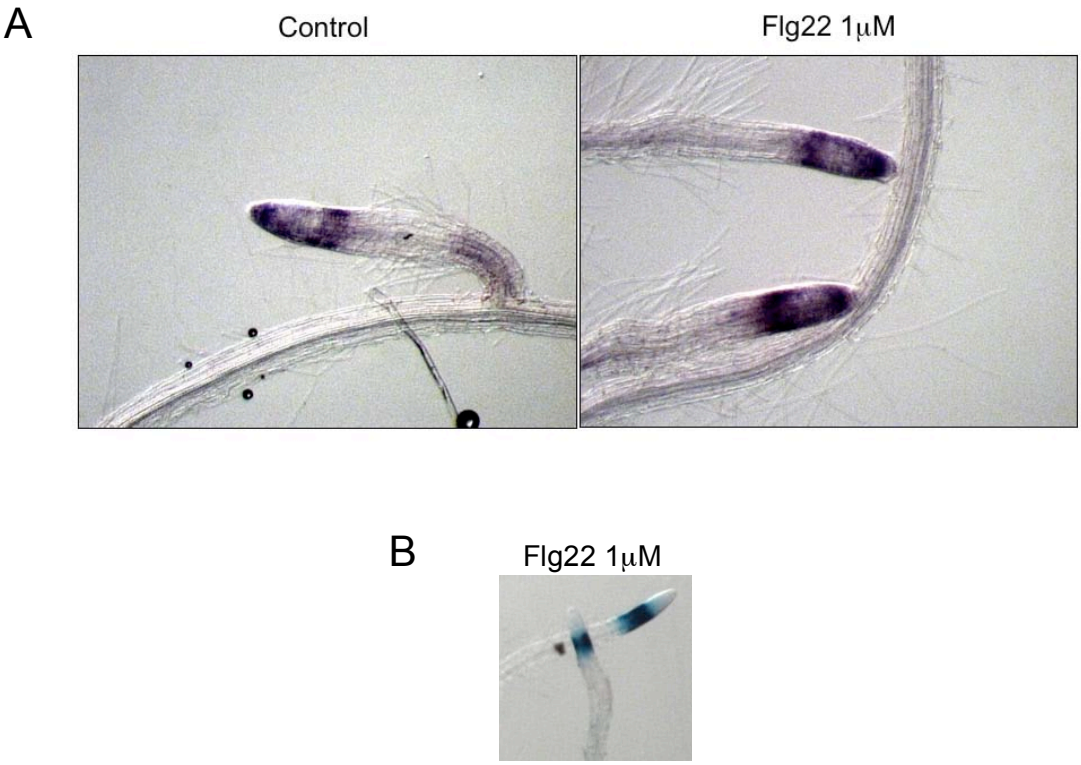

Figure 8. ROS detection by NBT staining in *Arabidopsis* roots after Flg22 treatment.
(A) 10 day-old Col-0 seedlings were treated with 1μM Flg22 for 5, 10, 20 and 30 minutes and stained with NBT. Similar results were obtained for all time points. The pictures shown correspond to the 20 minutes time point.
(B) 10 day old *CYP71A12*$_{pro}$*:GUS* seedlings treated with 1 μM Flg22 for 5h before GUS staining.

this question, ROS production in the roots of Flg22-treated *Arabidopsis* seedlings was observed by nitro blue tetrazolium (NBT) staining. No difference between the control and the Flg22-treated plants was observed (Figure 8). However, the roots naturally produce ROS at the root elongation zone, precisely where Flg22 signaling is occurring (cf. chapter 2) making it difficult to draw any definite conclusions. Production of ROS has been shown to be important for cell wall loosening during elongation in maize roots (Liszkay et al., 2004).

II.2 *CYP76C2* is an SA-dependent gene

To investigate the regulation of *CYP76C2* by SA, its expression was monitored in seedlings following Flg22 elicitation in various mutants impaired in SA production (*sid2-2*, *pad4-1*) and SA signaling (*npr1-1*). SID2 is an isochorismate synthase required for the production of SA (Wildermuth et al., 2001). *PAD4* encodes a lipase-like gene required for SA signaling upstream of SA production (Zhou et al., 1998). NPR1 is key transcriptional regulator of many SA-responsive genes and is required for the SA-mediated systemic acquired resistance (Cao et al., 1994). The SA signaling pathway is diagramed in Figure 9A. Interestingly, the activation of *CYP76C2* was reduced in the SA mutants *pad4-1* and *sid2-2* (Figure 9B). However, *CYP76C2* was expressed normally in the *npr1-1* mutant. These results show that *CYP76C2* is an NPR1-independent but SA-dependent gene. The SA dependency of *CYP76C2* was confirmed in adult leaves infected with *Pst*DC3000. Indeed, 24h after inoculation, compared to wild-type plants, *CYP76C2* expression was reduced in *pad4-1* and in *nahG* plants carrying the bacterial *nahG* gene coding a salicylate hydroxylase that converts SA into catechol (Delaney et al.,

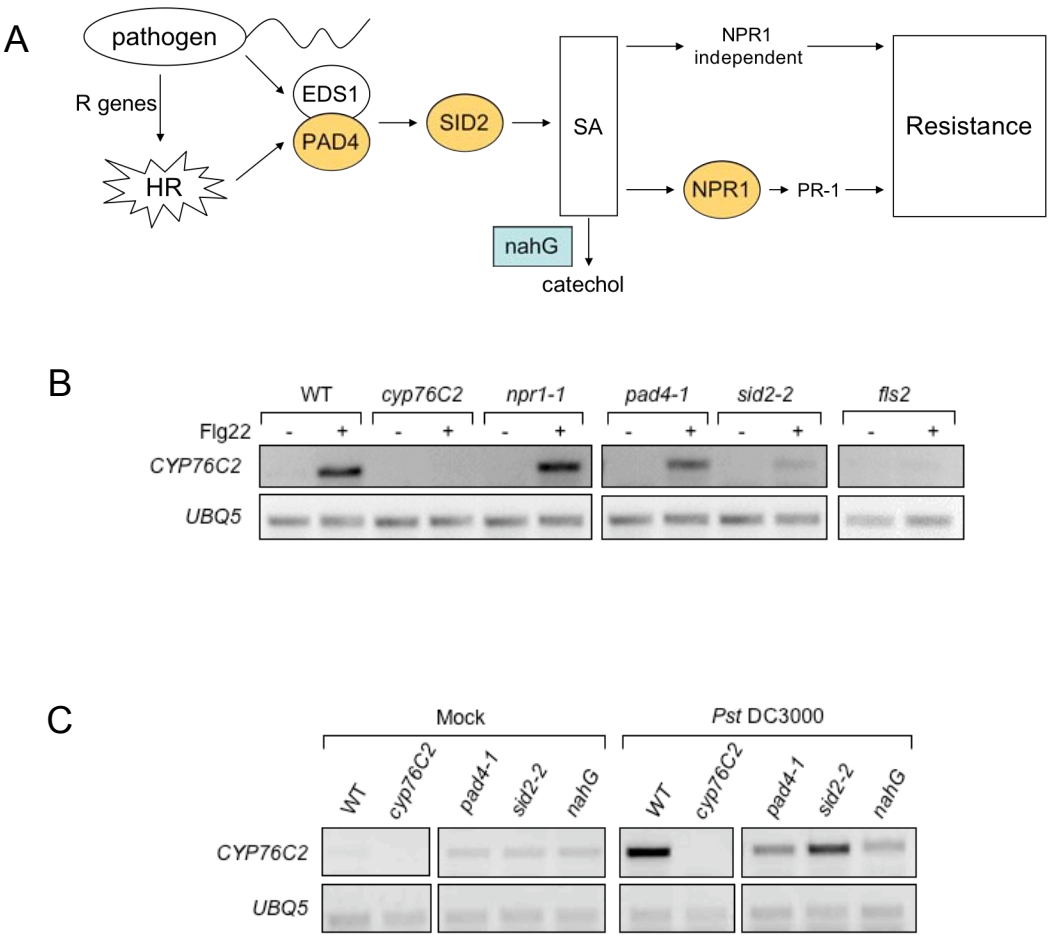

Figure 9. *CYP76C2* expression is SA-dependent.
(A) Simplified scheme of the SA pathway.
(B) RT-PCR in 10 day-old Col-0, *cyp76C2, npr1-1, pad4-1, sid2-2* and *fls2* seedlings treated with water or 1μM Flg22 for 3h.
(C) RT-PCR in 4 week-old leaves 12h after infiltration of water or a bacterial suspension of *Pst*DC3000 at an OD_{600} of 0.002.

1994) (Figure 9C). However, *CYP76C2* expression in *sid2-2* 4-week old leaves was not reduced as much as in *sid2-2* seedlings treated with Flg22 (Figure 9B). These experiments will need to be confirmed by qRT-PCR. The PAD4-dependency of *CYP76C2* expression after infection by *Pst*DC3000 was later confirmed by the publication of microarray data in *pad4* and *eds1* mutants infected with avirulent strains of *Pst*DC3000 (Bartsch et al., 2006). EDS1, an interacting partner of PAD4, is required for basal resistance against biotrophic pathogens and SA accumulation during HR (Aarts et al., 1998; Parker et al., 1996; Xiao et al., 2005).

II.3 Infection studies in *CYP76C2* loss of function and overexpressing lines

To determine if *CYP76C2* plays a role in resistance against pathogens, an insertion mutant of *CYP76C2* and a *35S-CYP76C2* overexpressing line generated by Sébastien Grec were tested for their resistance against *Pst*DC3000 in adult leaves. The overexpression of *CYP76C2* in the *35S-CYP76C2* transgenic plants was confirmed by RT-PCR (Figure 10A). No enhanced or reduced sensitivity to *Pst*DC3000 or *Pst/AvrRPT2* was detected in these lines (Figure 10B). The resistance of *cyp76C2* and *35S-CYP76C2* against *Botrytis cinerea* was also tested. Once again, no obvious phenotype was detected (data not shown). *CYP76C2* is part of an 8 member subfamily including one pseudogene. Moreover, *CYP76C2* is part of a cluster with 3 other *CYP76Cs*: *CYP76C1*, *CYP76C3* and *CYP76C4*. It is possible that the absence of phenotype observed for *cyp76C2* is due to redundant functions among these genes. Their expression patterns are, however, very different. Insertion lines are available for

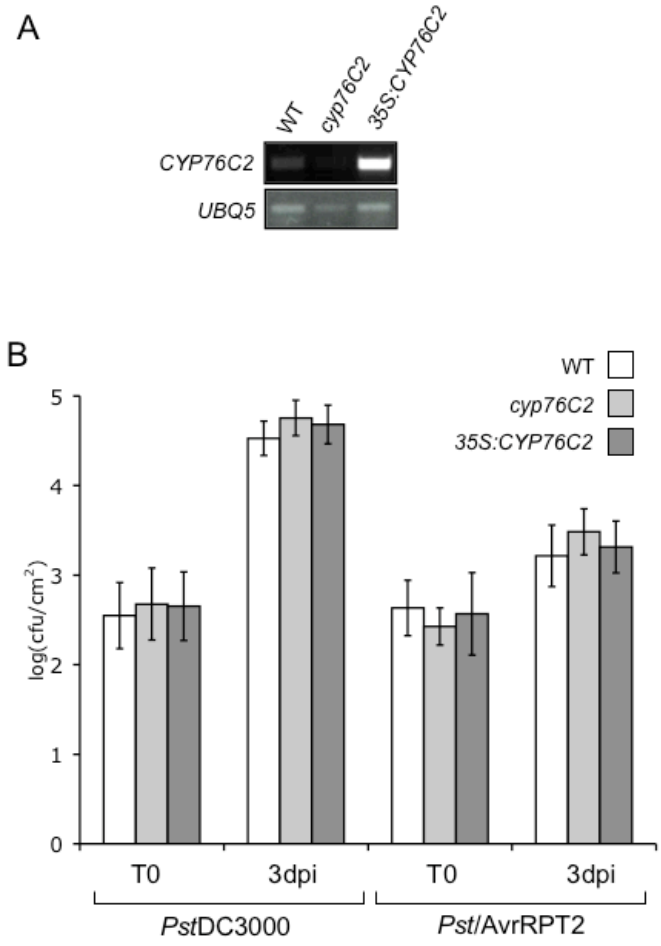

Figure 10. *CYP76C2* is not required for resistance against *Pst* DC3000.
(A) RT-PCR in 4 week-old Col-0, *cyp76C2* or *35S-CYP76C2* plants.
(B) growth of *Pst*DC3000 or *Pst*/AvrRPT2 in Col-0, *cyp76C2* and *35S-CYP76C2* plants. 4 week-old leaves were infiltrated at an OD_{600} of 0.0002 and bacterial titers were measured 3 days after infection. The data represent the mean ±SD of 6 replicates.

CYP76C1, *CYP76C3* and *CYP76C4*. Unfortunately, the fact that these genes are present in a cluster makes it almost impossible to obtain double or triple mutants.

Interestingly, three insertion lines for *CYP76C4*, the closest homolog to *CYP76C2*, showed a significant increase in susceptibility to *Pst*DC3000 (Figure 11) (this experiment was only carried out once and needs to be confirmed). However, it is unlikely that *CYP76C4* shares the same function as *CYP76C2 in vivo* since their expression patterns and the genes they are co-regulated with are totally different based on the Cytochrome P450 Expression Database CYPedia (www-ibmp.u-strasbg.fr/~CYPedia). Interestingly, CYP76C4 is predicted to be involved in the production of a sesterpenoid compound. The P450 CYP71D20 from tobacco was shown to catalyze two successive hydroxylations in the biosynthesis of the sesquiterpene capsidiol, a major antimicrobial in tobacco (Ralston et al., 2001). Therefore, it is possible that *CYP76C4* also plays a role synthesizing a terpenoid antimicrobial compound. This possibility will be further investigated via metabolic profiling in Strasbourg.

II.4 *CYP76C2* confers weak resistance against oxidative stress

CYP76C2 is expressed in all tissues undergoing cell death. Antioxidant production is a well-documented phenomenon during different types of programmed cell death including the HR and senescence (De Gara et al., 2003; Ge et al., 2007; Pavet et al., 2005; Woo et al., 2004). Moreover, SA is known to activate the production of antioxidants. In particular, SA activates many genes involved in the production of glutathione, a well-known antioxidant (Lieberherr et al., 2003; Rodriguez Milla et al., 2003). These antioxidants could participate in the control of cell death mechanisms. We

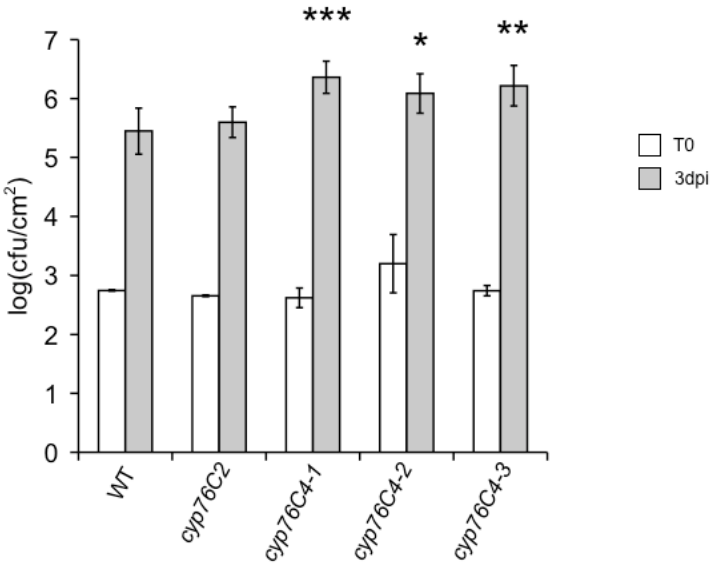

Figure 11. Insertion mutants in *CYP76C4* are slightly more susceptible to *Pst* DC3000. Growth of *Pst* DC3000 in Col-0, *cyp76C2* or *cyp76C4* insertion lines. 4 week-old leaves were infiltrated at an OD_{600} of 0.0002 and bacterial titers were measured 3 days after infection. The data represent the mean ±SD of 6 replicates. *P<0.05, **P<0.01, **P<0.001, two tailed *t* test.

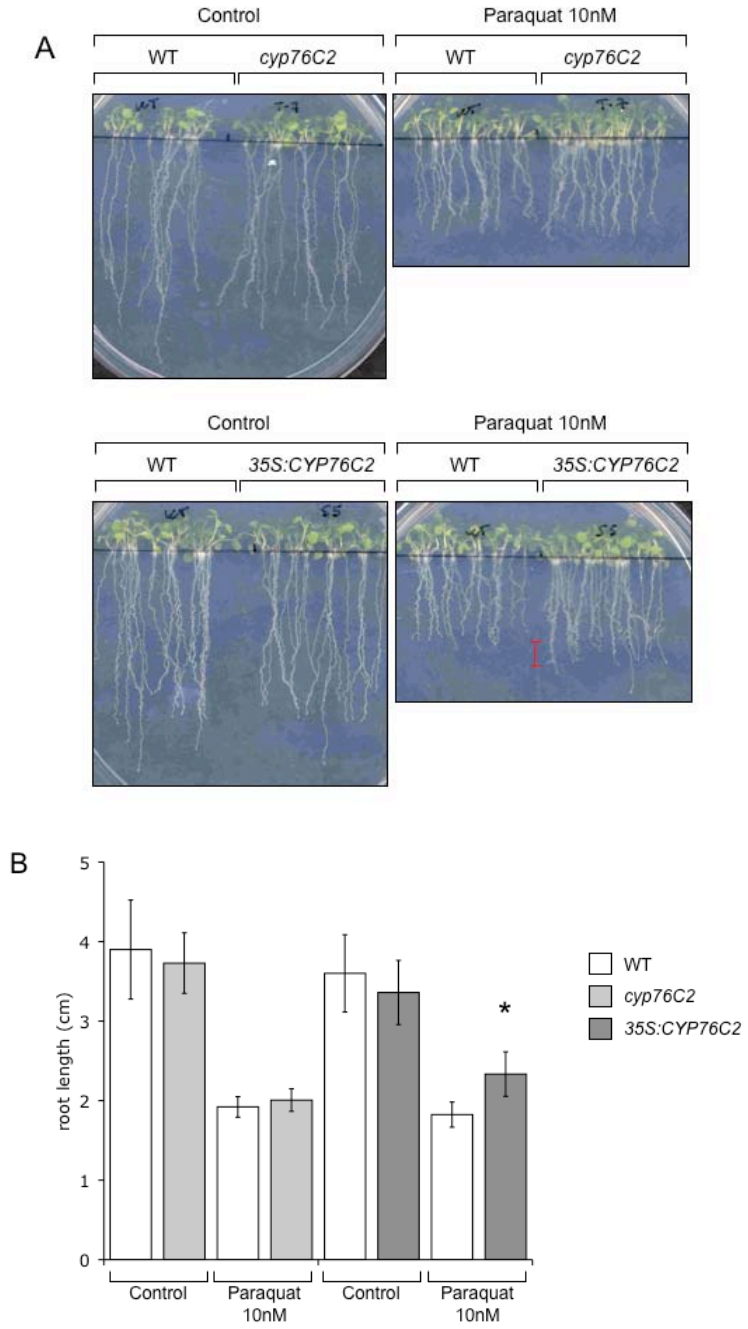

Figure 12. *CYP76C2* confers a weak resistance to oxidative stress.
(A) Col-0, *cyp76C2* and *35S-CYP76C2* seedlings germinated and grown for 12 days on vertical plates supplemented with 10nM paraquat.
(B) Root length of Col-0, *cyp76C2* and *35S-CYP76C2* germinated and grown for 12 days on vertical plates supplemented with 10nM paraquat. Data represent the mean ±SD. *P<0.05, two tailed *t* test.

investigated a potential role of *CYP76C2* in resistance against oxidative stress triggered by ROS production. Paraquat is a commonly used chemical to study oxidative stress. It catalyzes the formation of superoxide, a major ROS. The resistance of *cyp76C2* and *35S-CYP76C2* to oxidative stress was evaluated by measuring plant root growth on vertical plates containing 10nM of paraquat. A slight increase in paraquat resistance was observed in the case of *35S-CYP76C2* (Figure 12). This result was barely reproducible. Out of three experiments, only one showed a statistically significant difference between WT and *35S-CYP76C2* plants. However, all three experiments showed the same trend. This result suggests that *CYP76C2* may be involved in the biosynthesis of an antioxidant. However, additional experiments need to be carried out to confirm this result.

II.5 *CYP76C2* confers resistance against the phenylurea herbicide isoproturon

As mentioned before, P450s are known to detoxify various xenobiotics including pollutants such as herbicides. A close homolog of *CYP76C2*, *CYP76B1* from Jerusalem artichoke (*Helianthus tuberosus*), has been shown to actively metabolize phenylurea herbicides *in vitro* (Robineau et al., 1998). Moreover, *Arabidopsis* transgenic plants overexpressing *CYP76B1* are resistant to these herbicides (Didierjean et al., 2002). The detoxification of phenylurea herbicides by CYP76B1 is shown in Figure 13. During his post-doctorate, Sébastien Grec was able to show that CYP76C2 also metabolizes the phenylurea herbicide isoproturon *in vitro* by demethylation. However, no evidence was provided of this activity *in vivo*. As shown in Figure 14, *CYP76C2* does indeed confer tolerance to 1µM isoproturon *in vivo,* suggesting that isoproturon can be degraded *in vivo* by CYP76C2. Therefore, *CYP76C2* could potentially be used as a tool for

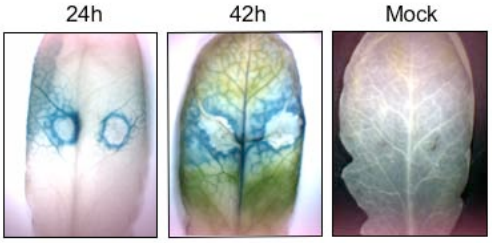

Figure 6. *CYP76C2* is induced around the lesion after infection by *Botrytis cinerea*.
GUS staining of 4 week-old *CYP76C2*$_{pro}$*:GUS* leaves infected by *B. cinerea*. A 5µL drop of a 5.10^5 spores/mL solution was placed on each side of the mid-vein. The GUS assay was performed 24h or 42h after infection.

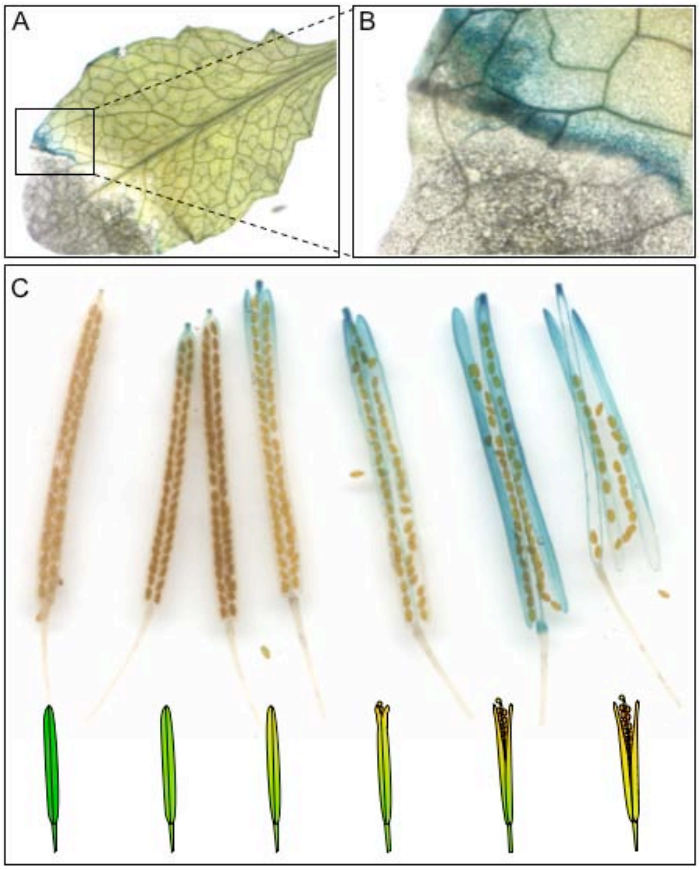

Figure 7. CYP76C2 is activated during senescence.
GUS staining in *CYP76C2*$_{pro}$*:GUS* senescing leaves **(A, B)** and opening siliques **(C)**.

phytoremediation by engineering plants resistant to that herbicide. The effect of CYP76C2 might even be synergistic. As PSII inhibitors, phenylureas are expected to trigger oxidative stress. Thus, in addition to herbicide detoxification, CYP76C2 may also support plant recovery via synthesis of an antioxidant compound.

IV. Discussion

Despite all the data presented in this chapter, no role for *CYP76C2* in plant defense could be found. Indeed, no phenotype of increased sensitivity or resistance to *P. syringae* or *B. cinerea* was observed for the *cyp76C2* insertion line or the *35S-CYP76C2* overexpressing line. CYP76C2 is part of an 8 members subfamily. Functional redundancy within this CYP subfamily could mask a potential phenotype for the *cyp76C2* insertion mutant. In addition, the *CYP76C2* gene is part of a tandem array including *CYP76C1*, *CYP76C3* and *CYP76C4,* rendering the generation of double and triple mutants problematic. On the other hand, functional redundancy within the CYP76C2 family seems unlikely since no other member of the CYP76C subfamily is co-regulated with *CYP76C2*. The *cyp76C2* and *35S-CYP76C2* lines were only tested for resistance against *P. syringae* and *B. cinerea,* but it is possible that CYP76C2 plays a role against a different pathogen or maybe an herbivorous insect. Moreover, the extreme virulence of *P. syringae* and *B. cinerea* on *Arabidopsis* plants could hide a potential mild phenotype of the *cyp76C2* or *35S-CYP76C2* lines. Testing the *cyp76C2* and *35S-CYP76C2* lines for resistance against other pathogens or herbivorous insects may provide us with a phenotype and prove *CYP76C2*'s involvement in plant defense.

In addition to its potential role in plant defense, the *in vivo* function of this P450 remains unknown. The gene is most highly expressed in flowers and maturing or germinating seeds during normal plant development. The fact that *CYP76C2* does not co-regulate with any obvious metabolic pathway based on the Cytochrome P450 Expression Database CYPedia (www-ibmp.u-strasbg.fr/~CYPedia) indicates its

involvement in a novel pathway and makes the functional characterization of this P450 challenging. Metabolic profiling of the *cyp76C2* insertion mutant and overexpressing line *35S-CYP76C2* will be performed at the Plant Molecular Biology Institute in Strasbourg in an effort to identify the metabolic pathway that *CYP76C2* is involved in. Identification of CYP76C2's substrate and product will then guide further investigations of the role of CYP76C2 in the plant defense response to pathogen attack.

Chapter 6. Conclusions and Future Directions

Research on plant innate immunity in the last fifteen years has led to great progress in our understanding of how plants recognize non-self and trigger defense mechanisms. It also revealed the complex virulence strategies that pathogens developed to circumvent these defense responses to establish a successful infection. In the vast majority of cases, these studies have been carried out using adult leaves or protoplasts. However, both adult plants and protoplasts have limitations as experimental systems, depending on the particular biological questions that are being asked. We therefore developed an assay based on *Arabidopsis* seedlings grown in liquid media, thereby providing the research community with a powerful tool to study plant-microbe interactions and the plant immune response to elicitors, as well as to perform genetic screens in a sterile, high throughput manner. In particular, this assay allowed me to extensively study the *Arabidopsis* response to MAMPs in the roots. Because of the extreme complexity of plant-microbes interactions in the rhizosphere, the role of roots in the plant defense response has been largely unexplored, despite the major impact that root colonization by microorganisms has on plant growth and defense against root pathogens.

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In Chapter 3, I used *promoter:GUS* reporters and the well-known MAMP-induced callose deposition assay to demonstrate that MAMPs trigger a strong response in *Arabidopsis* roots in very specific tissues, the elongation zone (EZ) for Flg22 and PGN or the mature zone of roots for chitin. These results raised the interesting hypothesis that plants have evolved tissue-specific MAMP responses to respond to different pathogens.

Further studies will need to confirm this hypothesis. First, other plants in addition to *Arabidopsis* will need to be tested to determine if MAMP-elicited tissue specific responses are conserved among different species, a project initiated by post-doctoral fellow Nicole Clay in the Ausubel laboratory. Second, more work needs to be done to determine whether MAMP signaling protects the plants against root pathogens and if the responses to various MAMPs have different effects on different pathogens.

I demonstrated in this thesis that MAMPs trigger the activation of the indole-glucosinolate biosynthetic pathway in *Arabidopsis* roots; in particular, the activation of the transcription factor *MYB51*, a major component of the MAMP-induced callose deposition response. Indole glucosinolates are a major class of defense compounds in *Brassicacae,* suggesting that the production and exudation of antimicrobials occurs in roots and shoots in response to MAMPs and could be the basis of PAMP/MAMP-triggered immunity (PTI). Evidence in support of this hypothesis has been obtained in unpublished work of Cristian Danna, a post-doctoral fellow in the Ausubel laboratory, who has found that MAMPs elicit the exudation of low molecular antimicrobial compounds from *Arabidopsis* seedlings.

As described in the general introduction to this thesis, suppression of PTI is generally considered to be a hallmark of successful pathogens. Consistent with this view, I showed that the MAMP response in *Arabidopsis* roots in suppressed by *P. syringae* in a coronatine- (COR) dependent manner. This is the first example of a defense mechanism suppressed by COR in plant roots. The development of the seedling assay and my studies on the COR-mediated suppression of MAMP signaling in roots gave me the opportunity to perform a forward genetic screen to identify new components in COR signaling

pathways. To date, 90 mutants impaired for the COR suppressive effect have been isolated. The identification of the corresponding genes in these mutants should help us to understand how COR suppresses PTI and could identify new players in JA signaling. In addition, this screen may also identify negative regulators of the MAMP response in roots.

Surprisingly, the beneficial microbe *P. fluorescens* WCS417r also suppressed the Flg22 response in roots, suggesting that suppression of PTI may be important for successful root colonization by beneficial microorganisms as well as pathogens. Investigating the impact of MAMP signaling on root colonization by beneficial or pathogenic microbes will help us to answer this question. Ethylene (ET) signaling plays a major role in potentiating the MAMP response in roots, as I showed in Chapter 3. Interestingly, a number of plant growth-promoting rhizobacteria (PGPRs) such as *P. fluorescens* produce the enzyme ACC deaminase, which degrades ACC, the precursor to ET. Several reports have shown the beneficial impact of this enzyme on root colonization and plant growth (Belimov et al., 2009; Gamalero et al., 2008; Wang et al., 2000). The ACC deaminase-mediated degradation of ACC could be one of the many mechanisms used by PGPRs to suppress the MAMP response in roots, thereby allowing successful colonization. In addition, many beneficial or pathogenic rhizobacteria or soilborne fungi produce phytohormones such as auxin, cytokinins, gibberellins and abscisic acid to promote plant growth, restructure root architecture or facilitate infections (Frankenberger, 1995). The production of some of those hormones could also be part of a strategy to suppress MAMP signaling and facilitate root colonization. Elucidating the potential role of ACC deaminase and various phytohormones in suppressing the MAMP

response in roots is a promising area of research and will most certainly help us to understand the molecular basis of root microbe-plant interactions.

This thesis also presented data concerning CYP76C2, an *Arabidopsis* cytochrome P450 that is highly induced by Flg22 and pathogens in leaves. I confirmed that this P450 is activated during cell death processes activated in response to pathogens as well as during senescence. This activation is partially dependent on the SA pathway and CYP76C2 could play a role in the production of an antioxidant to control oxidative damage during pathogenesis. More experiments need to be carried out to confirm the role of CYP76C2 in plant defense and in response to cell death. The unique expression profile of *CYP76C2*, which is not co-regulated with any obvious metabolic pathway, makes it extremely interesting, but also makes its functional characterization very challenging. Elucidating the function of CYP76C2 may be facilitated by metabolic profiling of the *CYP76C2* mutant and overexpressing lines that will be performed at the Institute of Molecular Biology in Strasbourg.

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Résumé

Au cours de l'évolution, les plantes ont développé des mécanismes de défense sophistiqués contre les pathogènes. L'une des premières lignes de défense se base sur la reconnaissance par la plante de motifs moléculaires très conservés associés aux pathogènes (PAMP/MAMP). Cette reconnaissance active divers mécanismes de défense, en particulier le dépôt de callose au niveau de la zone infectée. Malgré l'abondance des interactions racine-microbes, la réponse aux MAMPs dans cette partie de la plante reste largement inexplorée. Nous avons développé un système de culture hydroponique qui nous a permis d'étudier cette réponse chez *Arabidopsis thaliana* en se basant sur l'étude de lignées *promoteur:GUS* ainsi que sur le dépôt de callose. Nous avons trouvé que les racines répondent fortement aux MAMPs dans des régions bien spécifiques, en particulier dans la zone d'élongation. Cette réponse dépend de la voie de signalisation de l'éthylène, du facteur de transcription MYB51, du cytochrome P450 CYP81F2 ainsi que de la myrosinase PEN2. En outres, nous montrons que *Pseudomonas syringae* et *Pseudomonas fluorescens* sont capables de bloquer ce mécanisme de défense. En particulier, dans le cas de *P. syringae*, cette suppression s'effectue grâce à la production de coronatine (COR). L'action de la COR est dépendante de l'E3 ligase COI1 et du facteur de transcription JIN1/MYC2. Un screen génétique m'a permis d'isoler de nouveaux mutants incapables de bloquer la réponse aux MAMPs, dans le but d'identifier de nouveaux gènes impliqués dans la réponse à la COR. Enfin, ma thèse a porté sur l'étude du cytochrome P450 CYP76C2, fortement induit par les pathogènes. CYP76C2 est activé localement lors d'une infection par *P. syringae* ou *Botrytis cinerea* ainsi que lors des mécanismes de mort cellulaire. Je démontre que l'activation de CYP76C2 est partiellement dépendante de la voie de signalisation de l'acide salicylique et que ce gène est potentiellement impliqué dans le contrôle du stress oxydatif.

Mots clés: plante, racines, pathogènes, PAMP, callose, coronatine, cytochrome P450, stress oxydatif.

Abstract

Over the course of evolution, plants developed sophisticated defense mechanisms against bacterial and fungal pathogens. One of the first layers of plant defense is called PAMP triggered immunity (PTI) and is based on the recognition of conserved epitopes of pathogen-derived molecules called PAMPs/MAMPs (Pathogen/Microbe Associated Molecular Patterns). This recognition activates defense responses including the deposition of callose at the site of pathogen attack. Despite the fact that roots are the organs most subject to microbial interactions, MAMP signaling in roots remains largely unexplored. I developed an *Arabidopsis thaliana* seedling assay to study PTI in roots based on the detection of callose and the activation of *promoter:GUS* reporters of MAMP-responsive genes. I found that MAMPs trigger a strong response in roots dependent on ethylene signaling, the MYB51 transcription factor, the cytochrome P450 CYP81F2, and the PEN2 myrosinase, but independent of salicylic acid signaling. In addition, I show that the bacteria *Pseudomonas syringae* and *Pseudomonas fluorescens* suppress this response and that *P. syringae* is doing so by producing the phytotoxin coronatine. I found that coronatine acts via the E3 ligase COI1 and the transcription factor JIN1/MYC2. I performed a forward genetic screen to isolate mutants impaired in COR-mediated suppression in an attempt to identify new players involved in COR signaling. In this thesis, I also present data concerning *CYP76C2*, a gene encoding a cytochrome P450 that is highly induced by MAMPs and pathogens in *Arabidopsis* leaves. I confirmed that *CYP76C2* is activated during pathogen infection and various cell death elicited scenarios. Furthermore, I demonstrate that CYP76C2 is partially dependent on SA signaling and may be involved in controlling oxidative damage during infection.

Keywords: plant, roots, pathogens, PAMP, callose, coronatine, cytochrome P450, oxidative stress.

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