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Activation des voies de défense chez *Arabidopsis thaliana* par les oligogalacturonides et la flagelline

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Activation of *Arabidopsis thaliana* Defense Response Pathways by Oligogalacturonides and Flagellin

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Activation des voies de défense chez *Arabidopsis thaliana* par les oligogalacturonides et la flagelline

Mot-clé: MAMPs, oligogalacturonates, flagelline, Arabidopsis, pathogènes, réponse immunitaire, analyse du transcriptome, cytochromes P450, signalisation et glucosinolates

RESUME

Arabidopsis thaliana perçoit au travers de "pathogen- or microbe- associated molecular patterns" (PAMPs/MAMPs) l'attaque d'organismes pathogènes. De nombreuses études ont révélé des éléments communs dans les réponses de défense induites par les éliciteurs, mais les similarités et différences entre les voies de signalisation qu'ils induisent restent encore mal connues.

Les oligogalacturonates (OGs) sont des éliciteurs dérivant de la paroi des plantes qui induisent une large variété de réponse de défenses. Le transcriptome de plantules traitées par les OGs révèle une réponse transitoire de nombreux gènes, notamment une réponse transitoire et précoce de deux cytochromes P450 codant pour CYP81F2 et CYP82C3, Une analyse de l'expression de ces gènes dans divers mutants de défense suggère que leur réponse rapide à cet éliciteur est indépendante des voies de signalisation du salicylate (SA), du jasmonate (JA) ou de l'éthylène (Et). Ces gènes utilisés comme rapporteurs de la réponse au OGs, sont aussi induits par d'autre MAMPs, notamment les

lipopolysaccharides, la flagelline (Flg22), et la chitine.

Une analyse additionnelle du transcriptome a été menée avec des plantules traitées par deux éliciteurs très différents, les OGs, polysaccharides provenant de l'hôte, et Flg22, un peptide de synthèse dérivé de la flagelline bactérienne. Ces deux éliciteurs déclanchent des réponses similaires, rapides et transitoires. Elles sont caractérisées par l'activation précoce de voies de signalisation multiples, associées au JA en particulier. Cependant l'activation par Flg22 est plus forte, il induit un plus grand nombre de gènes. L'amplitude de l'activation est dose-dépendante dans les deux cas, mais, même aux plus fortes concentrations, les OGs n'induisent pas autant de gènes que Flg22. De plus, une activation des processus de sénescence, des voies de sécrétion SA-dépendante et de l'expression la protéine PR1 ne sont observés qu'en réponse à Flg22.

CYP81F2 participe à la réponse induite précoce aux eliciteurs (OGs and Flg22). Son expression est indépendante de SA, de JA et de l'éthylène. *CYP81F2* semble coder pour une indole glucosinolate 4-hydroxylase requises pour la formation de callose en réponse à Flg22.

Activation of *Arabidopsis thaliana* Defense Response Pathways by Oligogalacturonides and Flagellin.

Keywords: MAMPs, Oligogalacturonides, Flagellin, Arabidopsis, pathogens, immune response, transcript profiling, cytochromes P450, signaling and glucosinolate.

ABSTRACT

Pathogen attacks are perceived in *Arabidopsis thaliana* through recognition of pathogen- or microbe- associated molecular patterns (PAMPs/MAMPs). Although the study of various elicitors has revealed significant overlaps in defense response, the degrees of similarity/difference between MAMPs are not well defined.

Oligogalacturonides (OGs), plant cell wall-derived elicitors, induce a wide range of defenses responses. Transcript profiling of Arabidopsis seedlings treated with OGs indicates that the response to OGs involves a transient response in the regulation of many genes. Among the genes, two cytochrome P450s, *CYP81F2* and *CYP82C3,* are significantly induced shortly after OGs treatment. Monitoring the expression of these genes in a variety of defense-related mutants suggests that their rapid induction, mediated by OGs is independent of SA, JA, or Et signaling pathways. These reporter genes are also highly expressed in response to other MAMPs, including lipopolysaccharide (LPS),

flagellin (Flg22), or chitin.

Additional transcriptional analysis was carried out with OGs and pathogen-synthesized flagellin (Flg22), two very different elicitors. Both triggered a fast and transient response that are similar. This response is characterized by activation of the early stages of multiple defense signaling pathways, particularly JA-associated processes. However, the response to Flg22 is stronger in the number of genes differentially expressed and the amplitude of change. The magnitude of genes induction was in both cases dose-dependent, but, even at very high concentrations, OGs did not induce as many genes as Flg22. Moreover, activation of senescence processes, SA-dependent secretory pathway genes, and *PR1* expression was only observed with Flg22 elicitation. These results suggest a lower threshold for activation of early responses than for sustained late innate immune defenses.

Induction of the *Arabidopsis CYP81F2* gene is part of the early induced response to elicitors (OGs and Flg22). *CYP81F2* gene expression is independent of the SA-, JA- and Et-signaling pathways. CYP81F2 seems to catalyze the 4 methoxylation of indolic glucosinolates, which is required for callose formation in response to Flg22.

GLOSSARY

4MI3M	4-methoxy-indole-3-methyl-glucosinolate
AVR	Avirulence
Ca^{2+}	Calcium
CHS	Chalcone synthase
DAMPs	Danger associated molecular pattern
DP	Degree of polymerization
Et	Ethylene
Flg22	Flagellin peptide of 22 amino acid
GST	Glutathione-*S*-transferase
H_2O_2	Hydrogen peroxide
HR	Hypersensitive response
I3M	Indole-3-methyl-glucosinolate
ISR	Induced Systemic Resistance
JA	Jasmonic acid
MAPK	Mitogen-activated protein kinase
MAMPs	Microbe associated molecular pattern
LRR	Leucine-rich repeat
LPS	Lipopolysaccharide
NO	Nitric oxide
$O_2^{-\cdot}$	Superoxide radical
OGs	Oligogalacturonides
OH^-	Hydroxyl radical
PAL	Phenylalanine ammonia-lyase
PAMPs	Pathogen associated molecular pattern
PG	Polygalacturonases
PGIP	Polygalacturonases inhibiting proteins
PGN	Peptidoglycan
PR	Pathogenesis-related
RLK	Receptor like kinase
RLP	Receptor like protein
ROS	Reactive oxygen species
R protein	Resistance protein
SA	Salicylic acid
SAR	Systemic acquired resistance
TLR	Toll-like receptor
TFs	Transcription factors
TTE	Type three effectors
TTSS	Type three secretory system

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General Introduction

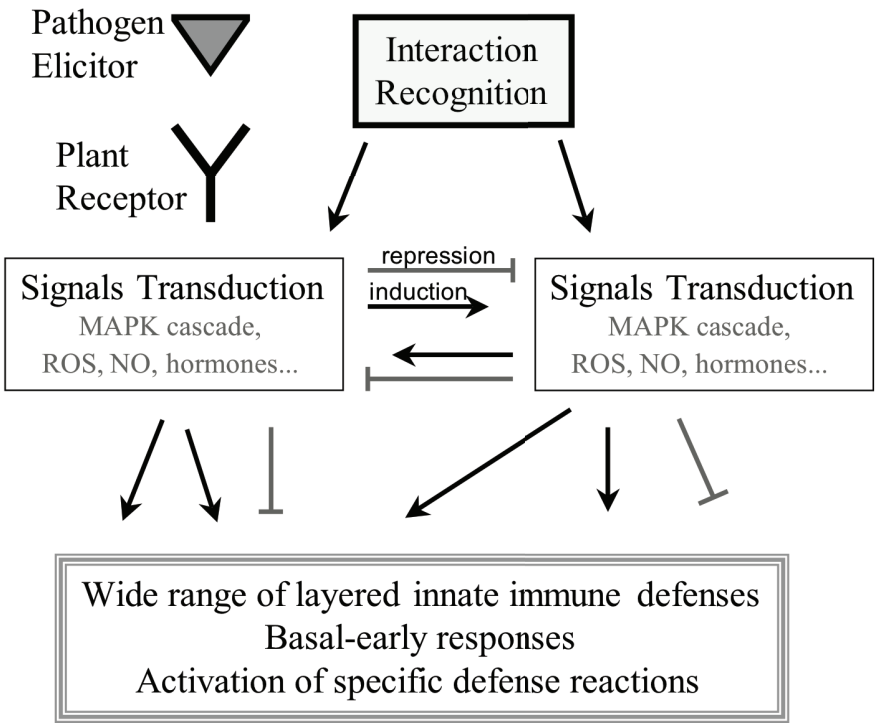

Figure 0-1: Simple scheme of the plant innate immune system.

The plant defense process relies on pathogen recognition and an efficient signaling network to activate basal responses and secondary specific defenses.

In nature, plants are continually exposed to a wide range of potential pathogens, including viruses, bacteria, fungi, oomycetes, nematodes, and insects. However, only a relatively small number of plant-microbe interactions lead to infection and disease. During evolution, plants have evolved sophisticated defense mechanisms to restrict pathogen invasion. These mechanisms are distinct from those of the vertebrate immune system. Unlike mammals, plants lack circulating immune cells and an adaptive immune response with immunological memory. The plant innate immune system relies on pathogen recognition by each cell and on signals emanating from the interaction of individual host cells with the pathogen. The detection of microbial pathogens plays a key role in the plant-defense response. An integrated signaling network downstream of pathogen recognition then mediates a layered response (Figure 0-1).

Overview of the complex network of defense responses

All of the defense mechanisms exhibited by *Arabidopsis thaliana* are similar to defense responses in other plants. The recognition of potential pathogens activates a complex network of signal transduction pathways. These pathways regulate multiple levels of plant innate immune responses. Many of these host defense events are triggered within minutes of pathogen perception. One of the first responses that can be measured is rapid activation of mitogen-activated protein kinase (MAPK) cascades, evolutionarily conserved signaling components in plants and animals. These MAPK cascades amplify

early responses and convert extracellular stimuli into defense responses against a wide range of pathogens (Asai et al., 2002; Colcombet and Hirt, 2008). MAPK cascades function via activation of three kinase modules (Zhang et al., 2006; Colcombet and Hirt, 2008) that involve sequential protein phosophorylation steps, leading to post-translational modification of hormone biosynthetic enzymes and transcription factors that regulate the expression of defense-related genes (Liu and Zhang, 2004) (Colcombet and Hirt, 2008).

Another early defense response is the production of reactive oxygen species (ROS), which not only play a key role as antimicrobial compounds, but also serve as key messengers in the plant defense response (Laloi et al., 2004; Pitzschke et al., 2006). Changes in concentration, the compartment in which the ROS are generated, and crosstalk between different reactive oxygen species (superoxide radical $O_2^{-\cdot}$, hydroxyl radical OH^-, and hydrogen peroxide H_2O_2), mediate a complex ROS- specific signaling response (Laloi et al., 2006). Considerable progress has been made regarding our understanding of the functions of the particular ROS agents including nitric oxide (NO). ROS, in particular H_2O_2 (Laloi et al., 2004; Pitzschke et al., 2006; Wang and Song, 2008) and NO (Neill et al., 2008), act as specific diffusible signaling molecules and contribute to the transmission of defense signals. They are also key regulators of physiological processes including stomatal closure (Desikan et al., 2004; Grun et al., 2006; Kotchoni and Gachomo, 2006). H_2O_2 and NO function in part by modulating the activities of downstream components in signaling, such as protein phosphatases, protein kinases, transcription factors (TFs), and calcium channels (Desikan et al., 2004).

In addition to ROS, other low molecular weight plant metabolites function as secondary-messenger molecules, including the plant hormones salicylic acid (SA), jasmonic acid (JA), and ethylene, which play key roles in plant defense-signaling networks (Oscar Lorenzo, 2003; Durrant and Dong, 2004). Roles for other plant hormones, such as abscisic acid (Mauch-Mani and Mauch, 2005), auxins (Navarro et al., 2006) and brassinosteroids (Nakashita et al., 2003), in the plant immune response are also emerging, but are not yet well understood. SA-, JA- and ET-dependent pathways are not necessarily activated by or effective against all pathogens. The plant's response often displays some degree of specificity toward particular classes of pathogens (De Vos et al., 2005; Glazebrook, 2005). Defense modules mediated by the signaling molecule salicylic acid (SA), such as production of pathogenesis-related protein 1 (PR1), are associated with resistance to biotrophic pathogens. In contrast, necrotrophic pathogens are more effectively resisted by those components of the defensive arsenal that are regulated via jasmonic acid (JA) and ethylene (ET) (Glazebrook, 2005).

Pathogens elicit a localized defense response at the site of infection that can be followed by a systemic acquired resistance (SAR) or induced systemic resistance (ISR). This secondary immunity in uninfected plant parts is effective against a wide variety of pathogens. SAR is triggered after infection by a necrotizing pathogen and is regulated by the SA signaling pathway (Durrant and Dong, 2004). ISR is activated by selected nonpathogenic bacteria independently of SA but requires JA and ethylene signaling (Feys and Parker, 2000; van Wees et al., 2000). Interestingly, the components of different hormone-dependent defense pathways can affect other signaling response pathways in

both synergistic and antagonistic ways (Feys and Parker, 2000; Spoel and Dong, 2008). Antagonistic interactions have been shown between the SA and JA pathways. On the other hand, plants are capable of expressing SA-, JA-, and ethylene-dependent defense responses at the same time without antagonistic effects (van Wees et al., 2000). Synergistic interactions between JA and ethylene signaling pathways have been reported, and result in the activation of genes encoding immune effectors, such as proteinase inhibitors and plant defensins (McDowell and Dangl, 2000; Oscar Lorenzo, 2003).

Upon pathogen recognition, the interplay of all these signaling events (MAPK cascade, ROS, NO, hormones, etc.) leads to a reprogramming of the plant transcriptome, physiological changes and activation of defenses (Figure 0-1). The ultimate goal is the production of defensive compounds and finally resistance. Cross-talk between different signal transduction pathways provides increased regulatory potential for activating multiple resistance mechanisms in varying combinations, and may help the plant to prioritize the activation of a particular defense pathway over another (McDowell and Dangl, 2000)

Plant defense - pathogen offense

As discussed above, most potentially pathogenic microbes are incapable of causing disease because plants have evolved multilayered strategies to limit and repel most attempted invasions. Effective protection against a particular pathogen by all accessions of a particular plant species is termed non-host resistance (Heath, 2000; Thordal-

Christensen, 2003; Nurnberger and Lipka, 2005). In this process the microbe is unable to overcome the preformed barrier defenses or the plant is able at a very early stage to detect the potential bioagressor and mount an effective response (Heath, 2000; Mysore and Ryu, 2004; Bent and Mackey, 2007). Non-host resistance is the most common type of resistance expressed by every plant. This robust protection has not been well explored because the symptoms or responses from the plant-pathogen interaction are not always visible and the underlying mechanism is still somewhat obscure.

- **Non-host resistance**

Although most recent studies, including this present work, focus on inducible defenses, the constitutive, preformed defenses are a major component of non-host resistance. These are the first lines of obstacles pathogens have to overcome, and include structural and chemical protection (Thordal-Christensen, 2003; Mysore and Ryu, 2004; Nurnberger and Lipka, 2005).

Morphological and structural features such as wax, cuticles and leaf hairs called trichomes cover the leaves. These barriers discourage insects from feeding on the leaf or depositing their eggs, and prevent fungi and bacteria from attaching and penetrating. The complex composition of the rigid cell wall provides an extra passive barrier against invaders. The cell wall is typically a matrix formed of cellulose fibers with proteins and high molecular weight polysaccharides such as pectin. The cell wall also serves as a reservoir of antimicrobial compounds, and is the site of pathogen recognition and a

source of defense signaling (Nurnberger and Lipka, 2005; Hernandez-Blanco et al., 2007).

A variety of plant produced substances act as protective agents, including preformed antimicrobial metabolites also referred as phytoanticipins, which are present prior to attempted infection. Chemical defenses are various and include antimicrobial peptides, proteins or secondary metabolites such as phenols, terpenoids, and isothiocyanates (Osbourn, 1996; Cowan, 1999; Heath, 2000; Nurnberger and Lipka, 2005). Some of these anti-microbial compounds are diffusible or volatile; however most of these compounds are stored in specific organelles, in specialized cells, e.g. trichomes, or in specialized cell-compartments (Osbourn, 1996). In general, phytoanticipins are toxic to a broad spectrum of insects, fungi, and bacteria. They can be directly toxic, or exist as conjugates such as glucosides. The conjugated glucosides become toxic after tissue disruption, which happens upon plant damage by insect or pathogen ingress, bringing the glucosides into contact with specific hydolyases that cleave off the sugar moieties. The sophisticated glucosinolate-myrosinase system is a chemical defense characteristic of brassicas, including *Arabidopsis*. The glucoside molecules and myrosinases can be localized in the same subcellular compartment of the same cells but in inactive forms, or they can be localized in different subcellular compartments of the same cell, or in different cells in *Brassica*. Loss of cellular integrity brings the glucosinolates and myrosinases to interact together, leading to the hydrolysis of the glucosinolate, resulting in release of glucose and an unstable aglycone. Subsequent rearrangement of the aglycone leads to the formation of a variety of bioactive products such as isothiocyanates, nitriles, etc., which can be

harmful to herbivores and pathogens (Grubb and Abel, 2006; Halkier and Gershenzon, 2006; Kissen et al., 2009).

In recent years, it has become more and more accepted that the ability of a plant to recognize a potential pathogen and mount an inducible "basal" defense response plays a key role in non-host resistance. The basal immune response is generally referred to as the innate immune response because of many similarities with the mammalian and insect innate immune responses that involve detection of microbes via Toll-Like pattern recognition receptors (Nurnberger et al., 2004; Ausubel, 2005; Zipfel and Felix, 2005). The term "innate immunity" in plants, however is not always well defined. Here the innate immune response will refer to the basal defense mechanisms that are activated in plants after perception of potential pathogens.

Microbes manage to overcome constitutive defensive layers in at least three ways: entering through wounds, invading through natural openings such as stomata and/or attacking directly the cell wall. Invaders can puncture cell walls with appressoria or digest cell walls where pectin is one of the first targets. As pathogens penetrate to the interior of the plant, they become subject to recognition. As in animals, pathogen attack is perceived through recognition of pathogen-derived or pathogen-host interaction-derived molecules; commonly called pathogen- or microbe- associated molecular patterns (PAMPs/MAMPs). PAMPs/MAMPs include molecules such as flagellin, peptidoglycan, or chitin from the microbe, as well as molecules derived from plant-microbe interactions such as oligogalacturonides derived from the hydrolysis of pectin by pathogen-encoded

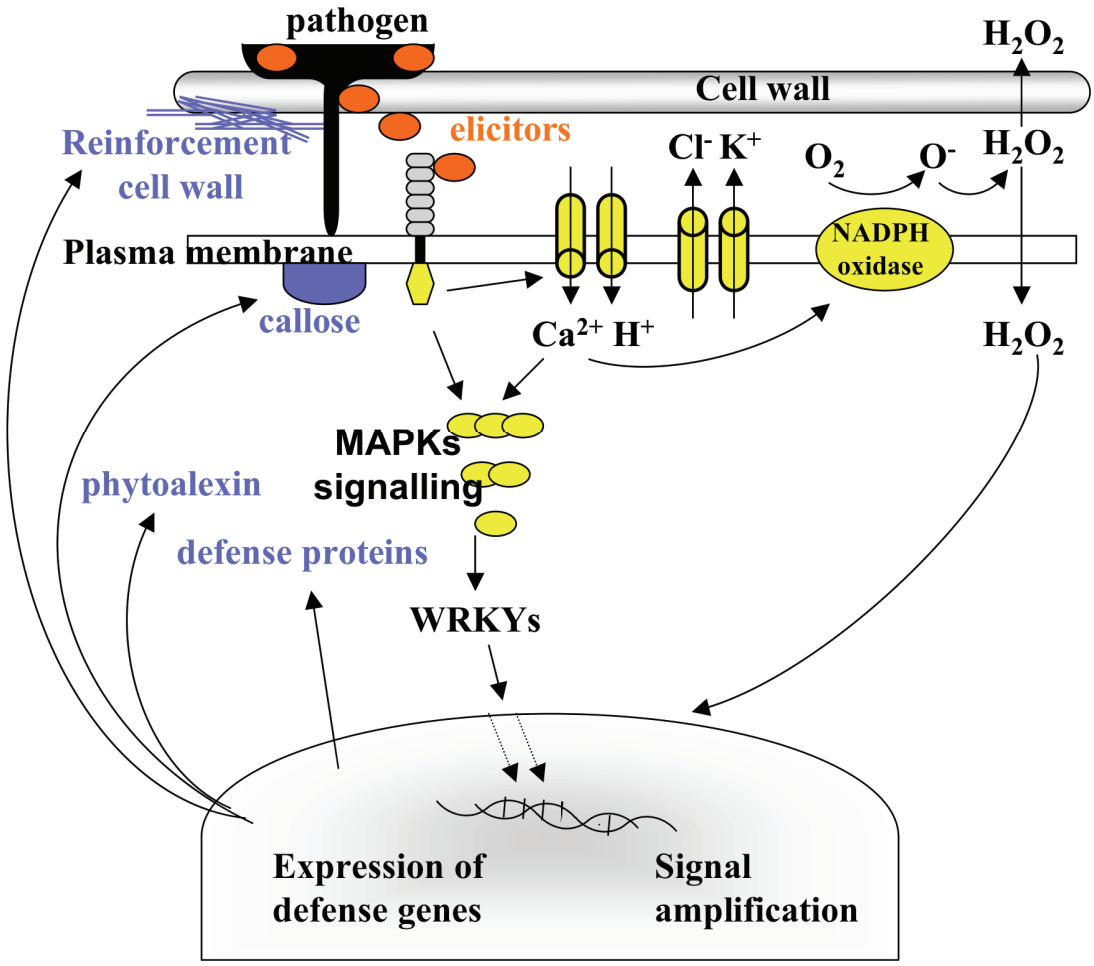

Figure 0-2: Model of plant inducible responses after recognition of a pathogen

In yellow are the rapid biochemical changes. In blue are long-term responses.

polygalacturonases. In general, MAMPs are detected by a suite of pattern-recognition receptors (PRRs). Microbial recognition is fundamental for triggering the inducible immune system (Gomez-Gomez, 2004; Ausubel, 2005; Jones and Dangl, 2006; He et al., 2007; Zipfel, 2008). In many plant-pathogen interactions, the responses to MAMPs are similar. Upon recognition, a wide range of physical, molecular, biochemical and chemical changes occurs in a sequential manner. They can be divided into two major categories: a general short-term response and a delayed long-term response. Of course, in between the plant defense machinery does not stop reacting and a complex network of signaling and transcriptional changes play an important role in transitioning from the short-term to the long-term responses. In general, the basal defense response elicited by PAMP/MAMP recognition appears to be sufficient to block most potential pathogens.

MAMPs/PAMPs initially induce many rapid biochemical changes (in yellow in Figure 0-2). Within minutes, there is an ion flux across the plasma membrane, transient increase of cytosolic Ca^{2+} concentration, alkalization of the apoplast, activation of MAPK cascades, generation of ROS and NO, and induction of ethylene biosynthesis (Dixon et al., 1994; Felix et al., 1999; Zhang and Klessig, 2001; Asai et al., 2002; Lecourieux et al., 2002; Kunze et al., 2004; Pemberton and Salomond, 2004; Zeidler et al., 2004). These early events are alarm signals, which are transmitted intracellularly. They activate a series of physical responses (in blue in Figure 0-2) such as the reinforcement of cell walls, by oxidative cross-linking of cell wall components, deposition of callose in the form of papillae at the site of pathogen invasion to block further penetration, and stomatal closure to prevent motile bacterial and fungal hyphae entry (Lee et al., 1999; Desikan et al.,

2004; Nurnberger et al., 2004; Melotto et al., 2006).

These early events are followed several hours later with additional toxic responses against pathogen invasion, some of which can damage the plant cells. This last arsenal of antimicrobial compounds that plants deploy includes defensins and pathogenesis-related (PR) proteins; lytic enzymes such as chitinases, glucanases, and proteases; and the biosynthesis of secondary metabolites called phytoalexins (Figure 0-2) (Dixon et al., 1994; Hahlbrock et al., 1995; Gomez-Gomez et al., 1999; Heath, 2000; Aziz et al., 2003; Nurnberger et al., 2004). Phytoalexins are low molecular weight antimicrobial chemical compounds that plants produce to combat infection. They act as toxins toward the attacking invader and often are targeted to a specific aggressor. Camalexin, an indolic compound, is the main phytoalexin found in *Arabidopsis thaliana (*Glawischnig, 2007). The biosynthesis of camalexin derives from tryptophan. The biosynthetic genes *CYP79B2, CYP79B3, CYP71B13* and *CYP71B15 (PAD3*) which encode proteins that catalyze this phytoalexin biosynthesis, are activated by pathogen infection (Schuhegger et al., 2006; Nafisi et al., 2007; Schuhegger et al., 2007). Interestingly, all the known genes involved in the camalexin metabolic pathway are cytochromes P450, which are discussed in detail in Chapter 3 of this thesis.

- **Host-pathogens**

In contrast to non-host pathogens, host-pathogens are microorganisms that successfully overcome the constitutive preformed and basal inducible defenses. These

successful pathogens gain access to the plant interior and deliver effector molecules that modulate host transcription, hormone signaling, and defense responses. Importantly, these effectors have the ability to suppress the basal inducible defense response, thereby allowing the pathogen to cause disease (Dangl and Jones, 2001; Hauck et al., 2003; Abramovitch and Martin, 2004; Li et al., 2005; Nomura et al., 2005; Chisholm et al., 2006; da Cunha et al., 2007; He et al., 2007). Pathogen-synthesized effectors that overcome basal defenses are an essential aspect of the pathogenic process.

The best studied pathogen effectors are ones that plant pathogenic bacteria deliver directly into host cells. These are called type III effector proteins (TTEs) (in some cases referred to as AVR or avirulence proteins). TTEs are injected via a proteinaceous pilus, the type III secretion system. Importantly, during the coevolutionary arms race between plants and their pathogens, plants have adapted their immune systems in order to detect these specific pathogen derived molecules or effectors (Figure 0-3). The bacterial effector proteins are recognized directly or indirectly by corresponding specific resistance (R) proteins. The recognition of TTEs by R proteins often leads to the so-called hypersensitive response (HR) to limit the pathogen spread. In this process, H_2O_2 functions as a signaling molecule that triggers the expression of defense genes and the activation of antimicrobial metabolites (McDowell and Dangl, 2000; Nurnberger et al., 2004). Delivery systems similar to the bacterial TTSS/TTEs are present in fungal (rust and powdery mildew) and oomycete pathogens. For example, haustoria forming fungal pathogens secrete effectors proteins into host cells to manipulate the host defenses and to harvest host nutrients. Many fungal/oomycete effectors have been identified in recent

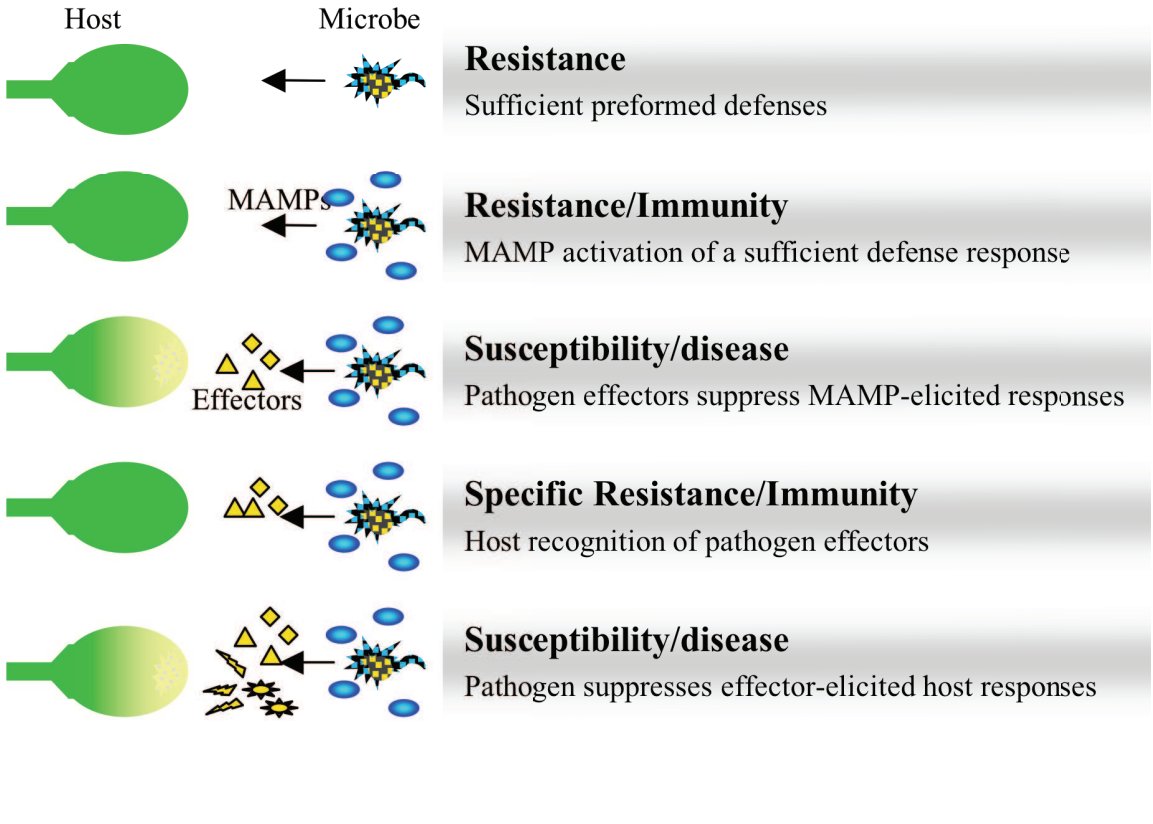

Figure 0-3: Different levels of plant immune defenses

years (Kamoun, 2006; Catanzariti et al., 2007; Birch et al., 2008; Micali et al., 2008). Interestingly, although the response to effectors is more aggressive than the response to PAMPs/MAMPs, both PAMP/MAMPs and effector proteins induce semi-redundant defense responses. Many of the inducible events are shared in host and non-host interactions (Heath, 2000; Dangl and Jones, 2001; Nurnberger et al., 2004; da Cunha et al., 2006), especially because MAMPs are present in all pathogenic and non-pathogenic microbes.

In host-pathogen interactions, resistance to infection depends on the plant defensive arsenal and the corresponding arsenal of pathogen virulence factors. For a pathogen to be successful, it has to evade the preformed defensive barriers, block the ability of the host to detect or respond to the potential invader by recognition of MAMPs, and suppress the HR response elicited by its effector proteins. That is, to be able to infect and cause diseases, the pathogen has to have the latest version of offensive weapons to inactivate the detectors that plants elaborate, and so on (Figure 0-3) (Thordal-Christensen, 2003; Nurnberger et al., 2004; Nurnberger and Lipka, 2005; Jones and Dangl, 2006; Bent and Mackey, 2007; Schwessinger and Zipfel, 2008). Such newly evolved pathogen race-specific virulence factors that counteract the function of R proteins have in turn driven the co-evolution of plant resistance genes and thus the development of pathogen race/plant cultivar specific disease resistance (Heath, 2000; Dangl and Jones, 2001; Kamoun, 2001).

MAMPs

It was discovered more than 30 years ago (Graham et al., 1977; Albersheim and Valent, 1978; Hahn and Albersheim, 1978; Roby et al., 1987) that plant defense responses could be activated by elicitors that signal the presence of a microbe. As discussed above, plants, like animals, have acquired the ability to recognize broadly conserved molecules characteristic of a given class of microorganisms that are not found in host cells. Remarkable similarities have been highlighted between plants and animals such as the types of MAMPs recognized, structures of their respective PRRs, and the activation of the basal responses (Gomez-Gomez and Boller, 2002; Nurnberger and Brunner, 2002; Ausubel, 2005; Nurnberger and Kemmerling, 2006). Researchers who study mammalian innate immune originally referred to pathogen-synthesized immune elicitors as "pathogen-associated-molecular-patterns" (PAMPs). However, because nonpathogenic microorganisms also produce "PAMPs", it make more sense to use the term MAMPs: microbe associated molecular pattern (Ausubel, 2005), which is gaining favor in the plant community.

MAMPs are evolutionarily conserved structures that have important roles in microbial physiology and are generally indispensable for microbes (Nurnberger and Brunner, 2002; Nurnberger et al., 2004). Many of these conserved pathogen components are recognized by the plant and trigger a defense response. The diversity of MAMPs is reflected by their various origins (bacteria, oomycete, fungi) and by the various biochemical families they belong to (oligosaccharides, peptides, proteins, glycoproteins,

sterols and liposacharides). Among examples of this diversity just in the oomycetes, a fragment of the calcium-dependent cell wall transglutaminase derived peptide (Pep13), elicitin protein (cryptogein), and hepta-β-glucans, a component of the cell walls, are all MAMPs that activate plant defenses. Elicitors recognized from bacteria can be extracellular components, such as the N-terminal domain of bacterial flagellin, lipopolysaccharides (LPS) that are abundant in the outer membrane of Gram-negative bacteria, and peptidoglycans (PGNs) from the cell-wall of Gram-positive bacteria. MAMPs can also be intracellular bacterial proteins, such as an N-terminal fragment from the elongation factor-Tu (EF-Tu). Components of fungal cell walls or membranes, such as chitin and ergosterol, also function as MAMPs that induce basal plant immune responses (Table 0-1) (Cote, 1998; Felix et al., 1999; Kamoun, 2001; Brunner et al., 2002; Lecourieux, 2002; Aziz et al., 2003; Kasparovsky et al., 2004; Kunze et al., 2004; Zeidler et al., 2004; Gust et al., 2007; Schwessinger and Zipfel, 2008). Elicitors from microbes are continuously being discovered and are not exhaustively cited here.

To activate the immune response, plants rely on the detection of at least one of these diverse MAMPs via a corresponding receptor. However our knowledge about MAMP-receptors is limited in contrast with the enormous list of microbial elicitors. Few MAMP receptors have been identified. Most identified MAMP receptors are transmembrane receptor-like kinases (RLKs). Interestingly, however, it has been shown that the heptaglucoside elicitor from *Phytophtora sojae* binds to an extracellular protein GBP (glucan-binding protein), which lacks intracellular signaling domains, as do the tomato xylanase receptor LeEIX1/2 and the rice chitin-binding protein CEBiP (Figure 0-4)

Elicitors	Origin	Biochemical type
transglutaminase (Pep13)	Oomycetes, cell wall	protein (peptide 13 a.a)
elicitin (cryptogein)	Oomycetes	protein
hepta-β-glucans	Oomycetes, cell wall	oligosaccharide
Flg22	Bacteria, flagellin	protein (peptide 22 a.a)
Lipopolysaccharide (LPS) (lipid A)	Bacteria, membrane	glycolipid
Peptidoglycan (PGN)	Bacteria, cell envelope	glycoprotein
EF-Tu (Elf18)	Bacteria, intracellular	protein (peptide 18 a.a)
chitin	Fungi, cell wall	oligosaccharide
ergosterol	Fungi, cell membrane	sterol
xylanase (EIX)	Fungi, pathogenicity factor	protein
Fumonisin B1	Fungi, phytotoxin	metabolite
Necrosis and Ethylene-inducing Peptide1 (Nep1)	Wide range	peptide
polygalacturonases	Fungi, pathogenicity factor	protein
oligogalacturonides	Plant, cell wall	oligosaccharide

Table 0-1: Elicitor diversity (danger-associated molecular patterns and microbe--associated molecular patterns) by their organism origin and biochemical type.

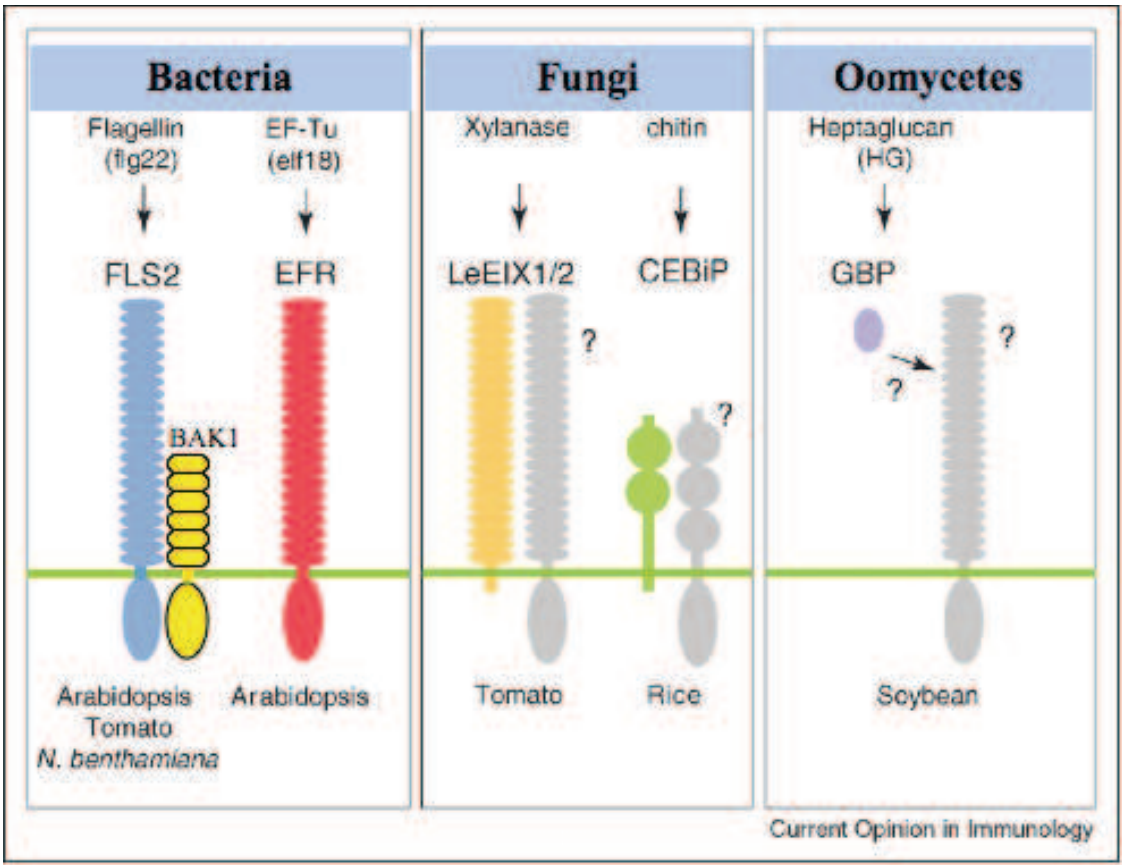

Figure 0-4: MAMP receptors.

Bacterial flagellin (Flg22) and EF-Tu (Elf18) are recognized by the LRR-RLKs FLS2 and EFR, respectively. Upon Flg22 recognition, FLS2 complexes with BAK1. Fungal xylanase is recognized by the RLPs LeEIX1 and LeEIX2. In rice, chitin binds to CEBiP, a transmembrane protein with two extracellular LysM domains. In legumes, the soluble glucan-binding protein (GBP) directly binds oomycetal heptaglucan. As LeEIX, CEBiP and GBP lack obvious signalling domains, they most likely interact with as yet unknown transmembrane proteins (in gray). (Adapted from Zipfel 2008)

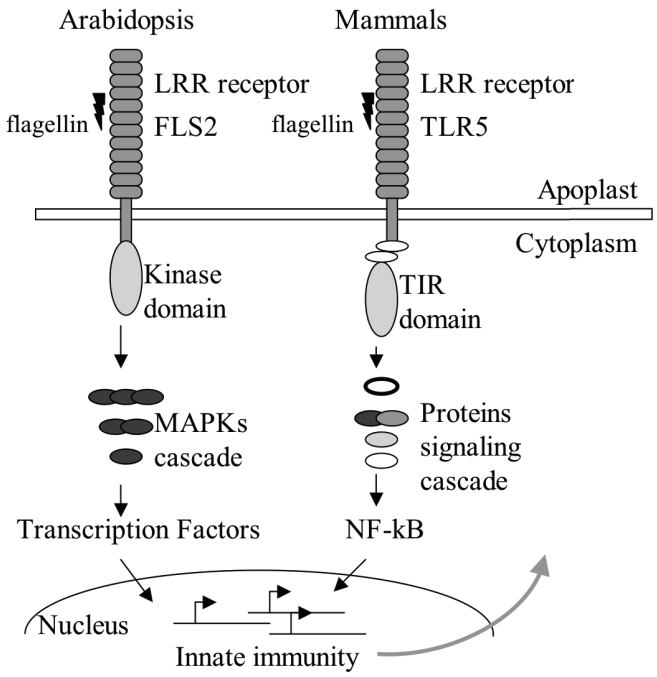

Figure 0-5: Common features of the plant and animal MAMP-perception.

Flagellin MAMPs/ PAMPs can be recognized by both Arabidopsis and mammalian LRR (leucine rich repeat) receptors.

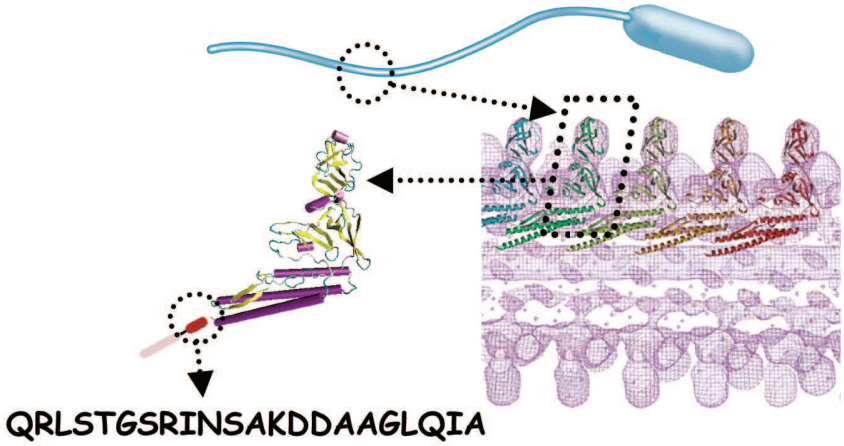

Figure 0-6: Flg22 is a synthetic polypeptide corresponding to a highly conserved 22-amino acid epitope from the bacterial flagellin N-terminus.

(Fliegmann et al., 2004; Ron and Avni, 2004; Kaku et al., 2006). It is not clear how these extracellular receptors transmit signals to induce defense, although they presumably interact with membrane-associated proteins to activate the basal defense responses.

Two *Arabidopsis* RLKs involved in bacterial MAMP perception have been well described: the Flagellin-Sensing 2 (FLS2) and EF-Tu receptor (EFR) (Gomez-Gomez and Boller, 2000; Zipfel et al., 2006), both of which contain extracellular leucine rich repeats (LRRs), a transmembrane domain, and a cytoplasmic serine/threonine protein kinase domain (Figure 0-4). The first plant MAMP receptor identified was FLS2. It was described as an RLK sharing homologies with animals and insect PAMP receptors. TLR (Toll-like receptors) also have extracellular LRR domains, and like FLS2, TLR5 is responsible for flagellin perception in mammals (Figure 0-5) (Gomez-Gomez and Boller, 2000, 2002). The flagellin/FLS2 model offers many advantages to study plant immunity in *Arabidopsis*. This elicitor can be easily synthesized and genetic tools such as *fls2* mutants are available.

Flg22 is a synthetic 22 amino acid polypeptide that is the best characterized MAMP in plants. Most plants recognize this highly conserved 22-amino acid epitope corresponding to eubacterial flagellin from *Pseudomonas aeruginosa* (Figure 0-6) (Felix et al., 1999). Flg22 triggers the production of an oxidative burst, alkalinization of the medium, activation of MAPK cascades, callose deposition, ethylene production, growth inhibition, and expression of pathogen-responsive genes such as *PR1, and PR5* (Figure 0-2) (Felix et al., 1999; Gomez-Gomez et al., 1999; Nuhse et al., 2000). Upon Flg22 perception, FLS2 activates MEKK1, MKK4/MKK5 and MPK3/MPK6 which in turn

activate the WRKY22/WRKY29 transcription factors (Asai et al., 2002) that transmit the Flg22-elicited signal to the nucleus.

The specific Flg22-binding site on FLS2 has not yet been identified. Upon Flg22 binding, FLS2 interacts with another RLK, BAK1 (Brassinosteroid hormone Receptor BRI1 Associated Kinase 1) and forms a complex that positively regulates Flg22 response (Chinchilla et al., 2007; Heese et al., 2007). This recent finding confirms that the perception of MAMPs is not as simple as a single receptor. Additionally, it has been shown that Elf18 (conserved peptide from EF-Tu protein) does not interact with FLS2, suggesting that individual MAMPs are specifically recognized by their cognate receptors.

Importantly, Flg22-treatment of Arabidopsis results in the restriction of the growth of the virulent bacterial pathogen *Pseudomonas syringae* pv. *tomato* strain DC3000, whereas *fls2* mutant plants show increased susceptibility to bacterial infection (Zipfel et al., 2004). Interestingly, Flg22-induced resistance was detected only when the bacteria were sprayed onto the leaf exterior and not when they were infiltrated directly into the apoplast. Perhaps the early detection of the first few bacteria entering into the leaf activates a defense response, including stomatal closure, before the pathogen can grow to a sufficient population to disrupt the host defense response. Although Flg22 perception and the downstream signaling pathways have been described in quite a bit of detail, it is still unclear how these various signaling pathways are integrated and how the mechanism of resistance is conferred.

Despite the advantage of the Flg22/FLS2 perception system as a model to dissect MAMP signaling, all elicitors do not signal via this canonical pathway. Some inducers of defense can be delivered directly by the microbe into the host in order to manipulate the plant metabolism. Sometime it is difficult to distinguish elicitors from pathogenic toxins. Indeed, some have the dual roles of being both a virulence factor and an elicitor of defense, which depends of the concentration of the elicitor, the host, the pathogen strain or other unknown conditions. For example, fumonisin B1 is a phytotoxin secreted by *Fusarium verticillioides* in its normal host maize, but this molecule activates plant defenses in Arabidopsis (Stone et al., 2000). In another example, Nep1 (Necrosis and ethylene-inducing peptide1)-like proteins are secreted by many pathogens which act as phytotoxic modulators of the host but also elicit immune responses (Pemberton and Salomond, 2004; Qutob et al., 2006). Finally, endopolygalacturonase1 (PG1) from the necrotrophic fungal pathogen *Botrytis cinerea*, functions as a pathogenicity factor by degrading the plant cell wall and elicits basal defense independently of its enzymatic activity (Poinssot et al., 2003). All these compounds are recognized by the plant as potential danger, that mediate defense reactions.

In addition to detecting microbes by recognition of non-self macromolecules, plants can also recognize infectious-self and/or modified-self molecules as MAMPs, which are also called danger-associated molecular patterns (DAMPs) (Schwessinger and Zipfel, 2008). Whereas most MAMPs are characterized by the fact that they are synthesized by microorganisms, but not hosts, DAMPs include also elicitors derived from the host itself. Oligogalacturonides (OGs), released from the plant cell wall by the action of fungal

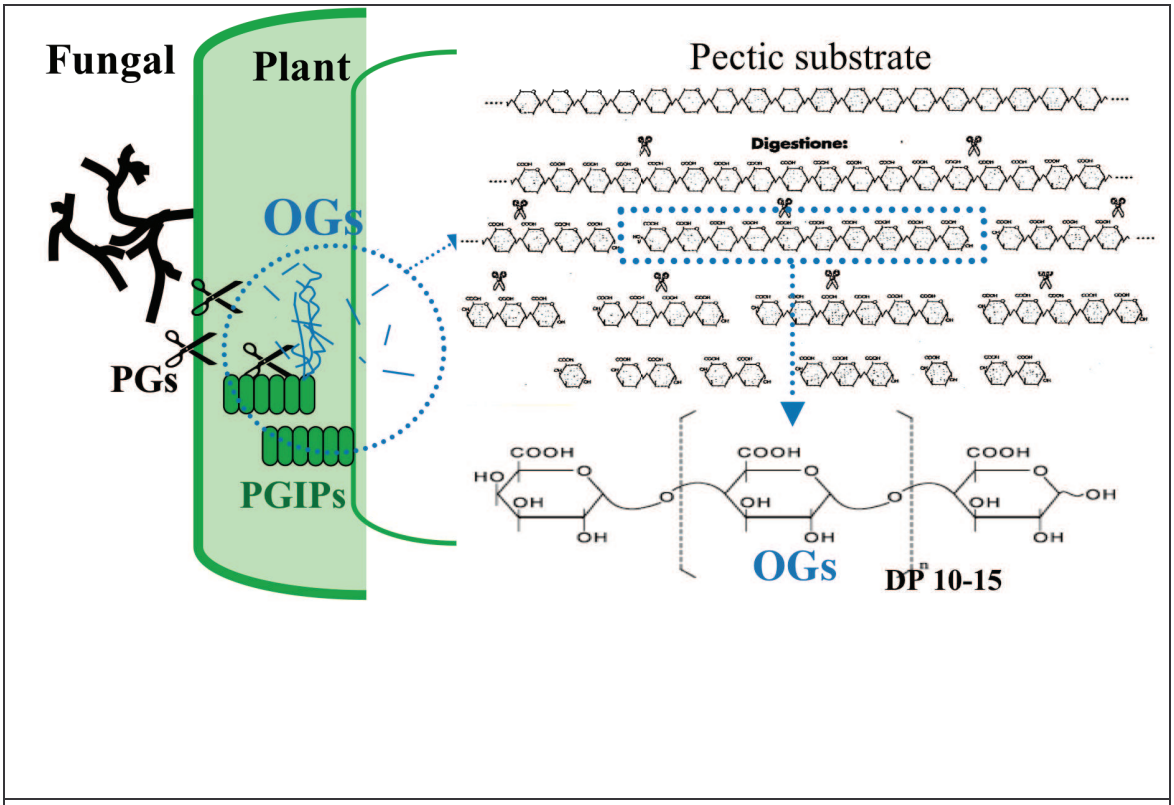

Figure 0-7. Oligogalacturonides (OGs) accumulation.

OGs accumulate in the plant cell walls as a result of the fungal hydrolytic enzyme poly-galacturonase (PG) and the plant PG inhibiting protein (PGIP) that prevents hydrolysis to monomers.
PGs cleave α-(1-4) linkages between D-galacturonic acid residues.

hydrolytic enzymes, are the best studied example of host-derived elicitors. As described in this thesis, OGs elicit a similar immune response as the MAMPs described above.

OGs attracted my attention, because they are the only plant-derived molecules known that could act as MAMPs and trigger immune responses. OGs are poly-α-(1→4)-D-galacturonic acid (linear chain), derived from homogalacturonan, a component of pectin in the cell wall (Figure 0-7). Pectic polysaccharides are digested by fungal polygalacturonase (PGs) as they penetrate into plant tissues. Interestingly, plants not only recognize OGs, but also the fungal PGs directly (Poinssot et al., 2003) and produce PG inhibiting proteins (PGIPs). PGIPs interact with PGs to slow down the degradation of the cell wall, resulting in the accumulation of active OGs elicitors (De Lorenzo and Ferrari, 2002; D'Ovidio et al., 2004). Most biological responses have been attributed to OGs with a degree of polymerization (DP) between 10 and 15, with no modification of the C terminus, or esterification (Ridley et al., 2001), or methylation (Navazio et al., 2002). OGs were first identified as an endogenous elicitor of phytoalexin accumulation in soybean (Hahn et al., 1981). They induce a variety of defense responses in common with other MAMPs, including an increase of cytosolic Ca^{2+}, production of an oxidative burst, and activation of MPK6 (Nuhse et al., 2000; Ridley et al., 2001; Poinssot et al., 2003; Aziz et al., 2004; Hu et al., 2004), similarly to Flg22. A variety of defense-related transcripts are upregulated in response to OGs, including β-1,3-glucanase, chitinases, phenylalanine ammonia-lyase (PAL), and glutathione-S-transferase (GST) isoforms in tobacco, parsley, soybean, carrots and Arabidopsis (Ridley et al., 2001; Aziz et al., 2004). Additionally, OGs elicit an acceleration of stomatal closing (Lee et al., 1999) and reduce

Botrytis cinerea lesion formation on grapevine leaves (Aziz et al., 2004). In plants, OGs do not play a unique role in the immune defense system, but also influence the growth and development of plant tissues (Ridley et al., 2001)

Similarly to the Flg22 and EF-Tu receptors, it was shown recently that OGs bind to an RLK protein. The extracellular domain of WAK1 (Wall-associated kinase 1) interacts with OGs in a calcium-induced change of conformation (Decreux and Messiaen, 2005; Cabrera et al., 2008). The receptor is a transmembrane protein containing a cytoplasmic Ser/Thr kinase domain. In contrast to *FLS2*, which is a single copy gene, *WAK1* belongs to a huge family of RLKs encoded in the *Arabidopsis* genome. The redundancy of the protein, its complex affinity with different pectic fragments, and specific binding condition, are many obstacles which could make it difficult to show that the WAK receptor is involved in OGs activated defense responses.

Although OG-mediated immune responses have been investigated in many crops species (Ridley et al., 2001), the fact that the OG perception system is not yet clear limits the progress that can be made in elucidating OGs-elicited signal transduction. Important gaps in our knowledge remain to be elucidated concerning the mechanisms that regulate and activate OGs basal responses.

In this thesis, I present work carried out in an *Arabidopsis* seedling-elicitor system to help elucidate a variety of aspects of OG-elicited defense signaling. Some of this work was carried out in collaboration with the laboratory of Giulia De Lorenzo at the

University of Rome. A major portion of my work involved full-genome transcriptional profiling of OG- and Flg22-treated seedlings. The defense responses elicited by OGs partially protect seedlings from *P. syringae* infection and activate genes independently of SA, JA, and ET signaling pathways. Furthermore, OGs-elicited transcript changes show significant overlap with *B. cinerea*- and Flg22- elicited transcriptional responses. Interestingly the late OGs and Flg22-responses show many differences. In addition to this work on OGs, I characterized the cytochrome P450 CYP81F2 that is involved in glucosinolate biosynthesis. CYP81F2-dependent responses are required for resistance to pathogens.

Chapter 1.

The Arabidopsis response to oligogalacturonides

ABSTRACT

Oligogalacturonides (OGs) are host-derived molecules that signal the possible presence of a pathogen and induce a variety of defense responses. They are released from plant cell walls by the action of degradative polygalacturonases produced by microbial pathogens. Transcript profiling of Arabidospsis seedlings treated with OGs for 1h or 6h was carried out using Affymetrix ATH1 full genome GeneChips using a seedling assay system (Chapter 1; Paper 1) (Songnuan et al., 2007). Analysis of the transcriptional profiling data indicates that the response to OGs includes changes in transcriptional regulation of a significant number of genes, but suggests that this response is transient. The importance of this response was confirmed by the finding that a substantial portion of the defense response to *B. cinerea* is mediated by OGs (Chapter 1; Paper 2) (Ferrari et al., 2007). In the pool of genes induced at 1h by OGs, 2 cytochomes P450, *CYP81F2* and *CYP82C3,* were among the most highly induced genes. Several genes regulated by OGs encode cythocromes P450, including *CYP71B15* (*PAD3*) and *CYP71A13* which are involved in camalexin biosynthesis (Zhou et al., 1999; Schuhegger et al., 2006; Nafisi et al., 2007) and *CYP76C2* which is expressed during the hypersensitive response to avirulent *Pseudomonas syringae* pv *maculicola* (Godiard et al., 1998). *CYP81F2* and *CYP82C3* were chosen as representative defense-related genes to further study the Arabidopsis response to OGs in seedlings. The perception of these oligosaccharide molecules is specific to the shoot part of the seedling and dependent on the particular structure, size and conformation of oligogalacturonic acid used to activate the response. Interestingly, OGs are primarily active in the seedling assay during the initial hour of

treatment, which is not a consequence of degradation by polygalacturonases. The transcriptional response to OGs returns almost to a basal level by 6h. Nevertheless, there is a significant correlation between transcriptional changes in OGs-treated seedlings and *Botrytis cinerea*-infected adult plants, indicating that a significant portion of the defense response to *B. cinerea* is mediated by OGs (Chapter 1; Paper 2) (Ferrari et al., 2007). Moreover, treatment of seedlings with OGs limits *Pseudomonas syringae* propagation, suggesting that the early inducible changes elicited by OGs are sufficient to protect the plant from pathogen attack. Additionally the rapid induction of genes by OGs is mediated by signaling pathways that are independent of salicylic acid, jasmonic acid, or ethylene (Chapter 1; Paper 3) (Galletti et al., 2008).

INTRODUCTION

Pathogenic microorganisms have to overcome preformed barriers to access the interior tissues of the plant. For successful penetration, microbial invaders produce and release hydrolytic enzymes that degrade host cell wall polymers. Among these, the most extensively studied are endo-polygalacturonases (PGs), which cleave the α-(1→4) linkages between D-galacturonic acid residues in non-methylated homogalacturonan polymers, a major component of pectin in the plant cell wall. To defend themselves from pathogen-encoded PGs, plants produce apoplastic proteinaceous PG inhibitors (PG inhibiting proteins (PGIPs)) that control and modulate the activity of PGs. As a consequence of the activity of the PGIPs, oligogalacturonides (OGs) with a degree of

polymerization between 10 and 15 accumulate (De Lorenzo and Ferrari, 2002). These pathogen-host interaction-derived OGs were identified as elicitors of defense responses in the early 1980's (Hahn et al., 1981). Early signaling events can occur within 20 minutes of OGs treatment and include an increase of cytosolic Ca^{2+}, an oxidative burst, and activation of MAP kinase activity (Lecourieux et al., 2002; Navazio et al., 2002; Aziz et al., 2004; Hu et al., 2004; Romani et al., 2004). Later in response to OGs, a variety of defense-related transcripts are induced. In Arabidopsis and grapevine, OGs induce many genes including those encoding glutathione-S-transferase (GST), phenylalanine ammonia-lyase (PAL), (Aziz et al., 2004; Hu et al. et al., 2004), and JA biosynthesis genes (allene oxide synthase) (Norman et al., 1999). In addition, OGs elicit an acceleration of stomatal closing in tomato, (Lee et al., 1999), phytoalexin accumulation in tobacco, castor bean, soybean and parsley (Davis et al., 1993; Mathieu et al., 1996; Ridley et al., 2001), and an increase in resistance to *Botrytis cinerea* in grapevine (Aziz et al., 2004). Although OGs-mediated responses in Arabidopsis have been investigated, the mechanisms through which OGs activate such responses remain unclear.

RESULTS

1- Transcriptional profiling of Arabidopsis seedlings treated with OGs

Although many early events have been identified following OG treatment of plants, gaps remain in our understanding of the complex network of interactions involved in this

defense response. Ferrari *et al.* (Ferrari et al., 2003) demonstrated that the gene encoding polygalacturonase inhibiting protein, *AtPGIP1*, is expressed within 1h of OG treatment of Arabidopsis. *AtPGIP1* transcript levels remain high for a few hours before returning to basal levels. To identify the full complement of Arabidopsis genes that are transcriptionally regulated in response to OGs, a full-genome expression profiling of Arabidopsis RNA isolated from OG-treated seedlings grown in liquid medium was carried out using Affymetrix ATH1 GeneChips in collaboration with Simone Ferrari at the University of Rome. The seedling system facilitates the study of defense signaling pathways, in part because the sterile conditions avoid opportunistic environmental infection that could obscure the specificity of the OG response. Moreover, the seedling assay is faster and more physiological than producing and treating protoplasts (Chapter 1; Paper 1) (Songnuan et al., 2007). Seedlings were grown in 12-well tissue culture plates (15/seedlings well) with 1ml of Murashige and Skoog Basal medium supplemented with 0.5% sucrose, pH 5.8. On the basis of previous results obtained with *AtPGIP1* (Ferrari et al., 2003), the seedlings were treated with 200µg/ml of OGs for 1 and 6 hours.

Chapitre 1; Paper 1

Songnuan et al., 2007

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) [1,2] or the synthetic flagellin peptide Flg22 [3] 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 [4,5]. 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.

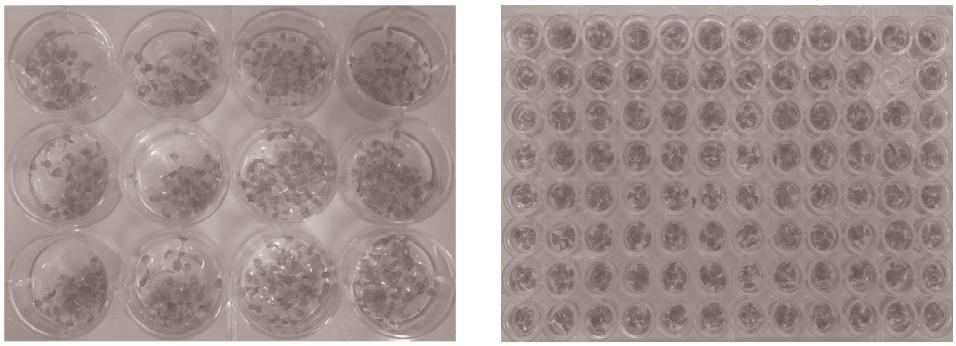

Figure 1. 10-day-old seedlings in 12-well (a) and 96-well (b) formats.

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* [6]. 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 [7]. 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"*)*.

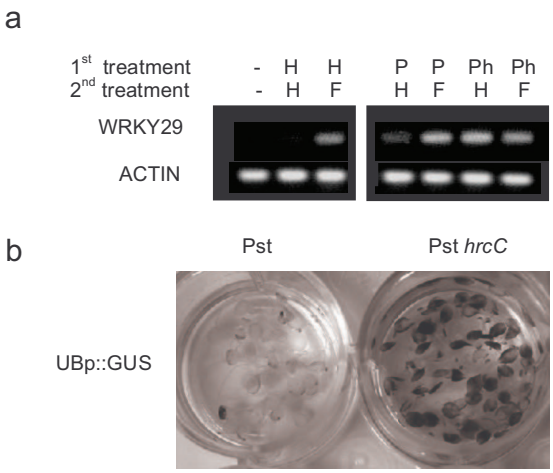

Figure 2. Using the seedling assay to study MAMP-mediated *WRKY29* gene induction and suppression by the type III secretion system of DC3000 (a). Seedlings were inoculated with bacteria or H_2O for 18 hours followed by elicitation with 1μL Flg22 or H_2O for 1 hour before tissue collection and RT-PCR. (- = 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.

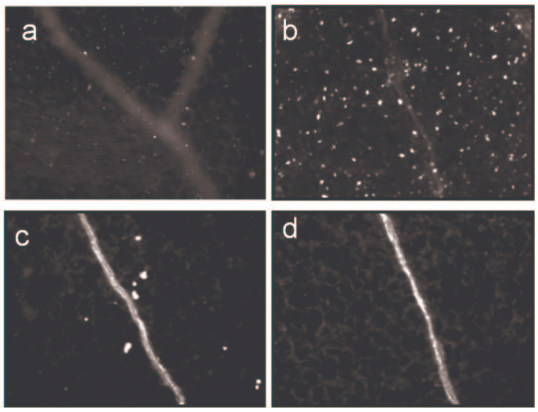

Figure 3. Callose deposition elicited by Flg22 can be detected in seedling cotyledons (a) wild-type Col-0 treated with water, (b) wild-type Col-0 treated with 1 μM Flg22, (c) *fls2* mutant treated with 1 μM Flg22 (d) *pmr4* mutant treated with 1 μM Flg22. Seedlings were stained with 0.01% aniline blue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

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[8]. No callose deposition was observed when a Flg22 receptor mutant (*fls2*) [8] (Figure 3c) or a callose synthase mutant (*pmr4*) [9] (Figure 3d) were treated with Flg22. Because the seedlings are in contact 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)* 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).

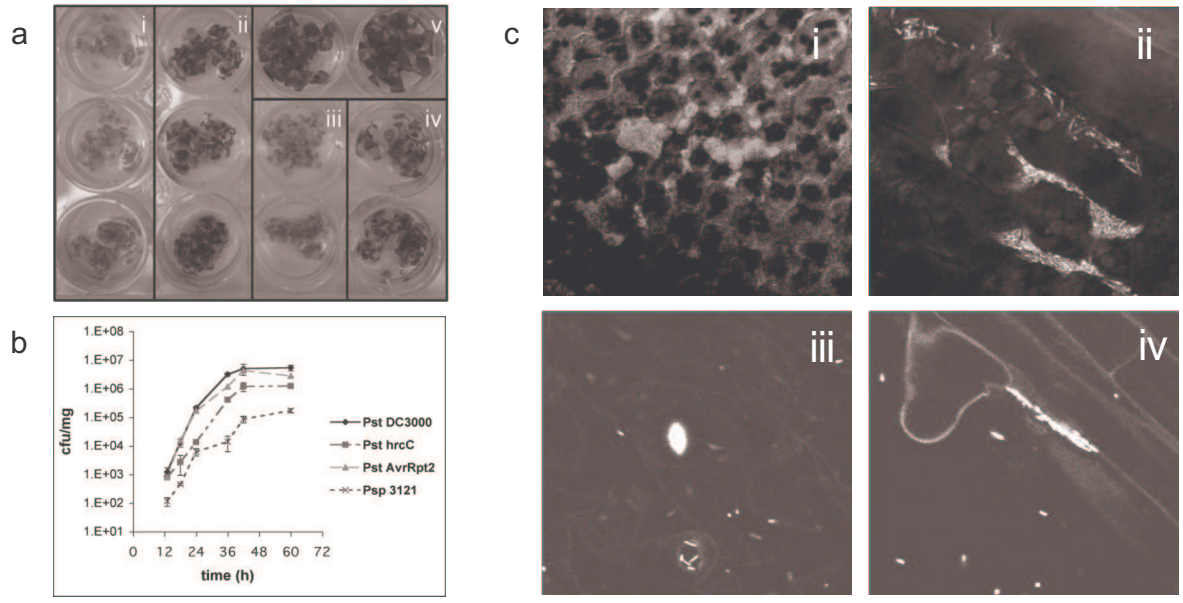

Figure 4. Seedlings infected with *Pseudomonas syringae*. (a) Seedlings exhibit different degrees of necrotic symptoms after 5 days of infection with wild-type *P. syringae* pv. *tomato* strain DC3000 (i), a type III secretion mutant *Pst* DC3000 *hrcC* (ii), *Pst* DC3000 carrying the avirulence gene *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).

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 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.* [10], 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 approach to show that seedlings pretreated with OGs were also significantly protected from DC3000 infection.

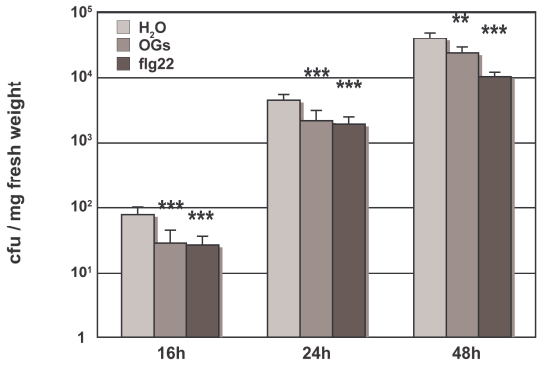

Figure 5. Flg22 and OGs protect seedlings from infection by *Pst* DC3000. 10-day old seedlings were treated with H_2O, 200 M OGs, or 1 M Flg22 8 hours prior to inoculation with DC3000 at OD_{600} = 0.0002. Bacteria inside the seedlings were counted at times specified. (** = $P < 0.01$, *** = $P < 0.001$,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

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 [11,12]. 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.

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.

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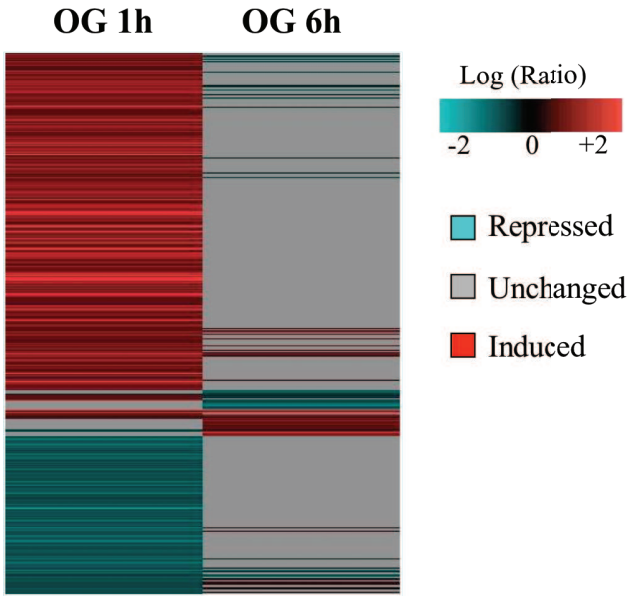

Figure 1-1: OGs induce a transient response.

Hierarchical clustering of 1872 genes that are differentially expressed in seedlings treated with oligogalacturonides for 1h or 6h versus untreated samples. Only genes with a probability of differential expression (P value) ≤ 0.001 and absolute change of 2-fold or more are included in the cluster analysis.

2- Transcriptional profiling analysis revealed a transient response

Total RNA was isolated from three replicates for each of the 1 and 6 hour treatments and analyzed using ATH1 DNA microarrays. After normalization, data for each treatment were combined to obtain mean expression values using the Rosetta Resolver v3.2 Gene Expression Data Analysis System (Rosetta Biosoftware, Seattle, WA, USA), as described in chapter 2 (Chapter 2; Paper 1 (Denoux et al., 2008)). The response to OGs included changes in transcript level for a significant number of the approximately 24,000 genes represented on the Affymetrix ATH1 GeneChip. As shown in the cluster matrix (Figure 1-1), most of the transcriptional changes occurred after 1h OGs treatment, whereas after 6h OGs treatment almost all genes returned to a basal level. After 1h of treatment, the expression of 1753 genes changed significantly (P-value $\leq$ 0.001) at least two fold compared to untreated samples. This included 1205 genes that were induced and 548 genes that were repressed. In contrast, only 185 genes were differentially expressed at 6h, including 107 genes that were induced and 78 genes that were repressed more than two fold (Figure 1-1). These data indicate that the major transcriptional response due to OG treatment of Arabidopsis seedlings occurs rapidly and declines after a few hours.

3- Identification of "marker" genes of the early response to OGs

Initially, we focused on genes significantly regulated by OGs that encode cytochromes P450 (Table 1-1) as reporters of significant metabolic changes, in order to

Name	Description	1h OG		6h OG	
		Fold Change	P-Value	Fold Change	P-Value
CYP82C3		34.59	0	1.01	0.99
CYP81f2	Involved in glucosinolate metabolism	33.58	0	-1.46	0.08
CYP71B15, PAD3	Camalexin indole phytoalexin biosynthesis	21.45	5E-22	-1.49	5E-03
CYP94C1	Omega-Hydroxylation of hydroxy-fatty acids, Induced in response to wounding and jasmonic acid.	17.01	3E-16	-1.14	0.65
CYP81D8		9.75	2E-24	-2.48	3E-03
CYP94B3		7.08	1E-09	4.47	0.02
CYP76C2	Expressed during hypersensitive response to pathogens, senescence and after wounding	6.34	5E-04	1.86	0.56
CYP96A4		5.13	3E-05	2.42	0.17
CYP707A3	ABA 8'-hydroxylase	4.97	2E-13	-1.25	0.83
CYP83B1	Biosynthetic pathway of indole glucosinolates	4.55	0	1.26	3E-05
CYP94B1	Long chain fatty omega-alcohol oxidases, regulated by pathogenic attack	4.46	3E-04	2	0.19
CYP710A1	C22-sterol desaturase	4.22	2E-13	2.42	0.03
CYP706A2		4.02	0	-1.04	0.55
CYP73A5	C4H cinnamate-4-hydroxylase	3.56	0	1.13	0.37
CYP71A12		3.55	0.04	3.87	1E-04
CYP71B5		3.46	4E-42	-1.21	0.38
CYP71B22		3	6E-06	-1.15	0.59
CYP81G1		2.91	4E-05	1.91	0.04
CYP71B13	Camalexin biosynthese	2.89	4E-03	1.81	0.29
CYP71B6		2.37	2E-15	1.71	1E-05
CYP98A3	C3H, encodes coumarate 3-hydroxylase	2.24	0	1.11	0.21
CYP71A16		1.17	0.41	4.95	6E-07
CYP705A5		-1.21	0.06	4.44	2E-22
CYP708A2	Thalianol Hydroxylase	-1.38	0.06	3.81	2E-05
CYP81F4		-1.78	5E-07	2.39	1E-22
CYP81H1		-1.94	3E-03	2.77	1E-04
CYP71B2		-2.09	2E-11	1.12	0.17
CYP96A1		-2.13	4E-04	-1.59	6E-03
CYP96A12		-2.15	2E-16	-1.08	0.56
CYP86A1	Fatty acid (omega-1)-hydroxylase	-2.37	9E-09	1.35	2E-03
CYP79F1	Biosynthesis of aliphatic glucosinolates	-2.74	3E-03	1.3	0.1
CYP71B26		-2.86	3E-28	1.22	2E-03
CYP83A1, EDR5, REF2	Glucosinolates biosynthesis	-2.91	1E-33	1.08	0.32
CYP89A2		-2.92	1E-26	1.04	0.75
CYP710A2	C-22 sterol desaturase	-3.12	1E-28	-1.03	0.51

Table 1-1: Cytochrome P450s differentially expressed after OGs 1 or 6 h treatment

Fold change and P-value are highlighted in gray if the probability for differential expression did not meet the threshold of P-Value<=0.01

identify possibly novel pathways involved in signaling or synthesis of defense compounds. P450s, which are characterized by a common heme-binding catalytic center, are involved in the biosynthesis or catabolism of a wide range of molecules, including structural macromolecules such as lignin and cutin, hormones, pigments, defense compounds, and xenobiotics. Only 207 cytochrome P450 genes are represented on Affymetrix ATH1 chips out of a total of 272 in the Arabidopsis genome. After 1h or 6h OGs treatment, the expression of 35 P450s changed 2 fold or more with a P-value of 0.001 or less (Table 1-1). Twenty genes from this family are significantly up-regulated and 11 P450s are significantly down-regulated after 1h treatment. Some of the P450s that are differentially expressed after OGs treatment are known to have a role in plant defense (Table 1-2). For example, CYP71A13 and CYP71B15 (PAD3) catalyze, respectively, early and final specific steps in the synthesis of camalexin, an indole-containing phytoalexin (Zhou et al., 1999; Schuhegger et al., 2006; Nafisi et al., 2007), CYP83B1 is a component of the indole glucosinolate biosynthetic pathway (Bak et al., 2001; Hansen et al., 2001), and *CYP76C2* is expressed during the hypersensitive response to avirulent *Pseudomonas syringae* pv *maculicola* (Godiard et al., 1998). The three most highly expressed CYP genes were *CYP81F2* (At5g57220; 33.58 fold); *CYP82C3* (At4g31950; 34.59 fold); and *CYP71B15* (At3g26830 21.45 fold). When these studies were initially carried out, CYP81F2 and CYP82C3 had not yet been assigned functional roles.

4- Changes in gene expression in Arabidopsis seedlings treated with OGs or in plants inoculated with *B. cinerea*

Name	Gene Description	1h OG		6h OG		References
		Fold Change	P-Value	Fold Change	P-Value	
CYP81F2	Involved in glucosinolate methabolim	33.58	0	-1.46	0.08	Clay et al. 2008
CYP71B15, PAD3	Last step of camalexin biosynthesis	21.45	5E-22	-1.49	5E-03	Schuhegger et al. 2006
CYP94C1	Fatty acid (omega-1) hydroxylase activity . Induced in response to wounding and jasmonic acid.	17.01	3E-16	-1.14	0.65	Kandel et al. 2007
CYP76C2	Expressed during hypersensitive cell death.	6.34	5E-04	1.86	0.56	Godiard et al. 1998
CYP83B1	Biosynthesis of indole glucosinolates	4.55	0	1.26	3E-05	Bak et al. 2001
CYP94B1, MLE2.8	Long chain fatty omega-alcohol oxidases, regulated by pathogenic attack	4.46	3E-04	2	0.19	Shilan et al 2007
CYP71B13	Camalexin biosynthesis	2.89	4E-03	1.81	0.29	Nafisi et al. 2007
CYP98A3	C3H, deposition of an unusual lignin	2.24	0	1.11	0.21	Schoch et al. 2001
CYP705A5	Thalianol pathway, triterpene synthesis	-1.21	0.06	4.44	2E-22	Field, et al. 2008
CYP708A2	Thalianol Hydroxylase	-1.38	0.06	3.81	2E-05	Field, et al. 2008
CYP86A1	Fatty acid (omega-1)-hydroxylase, root specific, suberin biosynthetic process	-2.37	9E-09	1.35	2E-03	Benveniste et al. 1998
CYP79F1	Biosynthesis of aliphatic glucosinolates	-2.74	3E-03	1.3	0.1	Chen et al. 2003
CYP83A1, EDR5, REF2	Biosynthesis of aliphatic glucosinolates	-2.91	1E-33	1.08	0.32	Hemm et al. 2003

Table 1-2: Cytochrome P450s involved in defense

Fold change and P-value are highlighted in gray if the probability for differential expression did not meet the threshold of P-Value<=0.01

In collaboration with Simone Ferrari, we addressed the physiological relevance of OGs-mediated defense responses in Arabidopsis seedlings by comparing the OGs response in seedlings with the response to the necrotropohic fungal pathogen *Botrytis cinerea* in adult plants. The rational for carrying out this comparison was that PGs are important virulence factors for *B. cinerea* and that Arabidopsis plants overexpressing PGIPs are more resistant to *B. cinerea* infection (Ferrari et al., 2003) while Arabidopsis PGIP antisense line are more susceptible to *B. cinerea* infection (Ferrari et al., 2006). We carried out full-genome expression profiling using Affymetrix ATH1 GeneChips on RNA isolated from 4-week old plants inoculated with *B. cinerea*. We determined the degree of overlap between gene expression changes induced by the fungus at 18 and 48 hours and those induced by OGs after 1 and 3 hours of treatment (Chapter 1; Paper 2) (Ferrari et al., 2007). This latter seedling OG profiling experiment was carried out subsequently to the 1 and 6 hour profiling experiment described in this chapter and is described in detail in chapter 2. The profiling data from chapter 2 were used for the comparison with the *B. cinerea* data because after 3 hours of treatment with OGs more genes were identified as differentially expressed than after 6 hours of treatment.

B. cinerea elicited a stronger transcriptional change at 48h post-inoculation (hpi) than at 18h. At 48hpi, when typical water-soaked lesions were visible, the expression of 4813 genes changed (at a P-value ≤ 0.001) at least two fold compared to uninfected plants; 1942 genes were up-regulated and 2871 were down regulated. At 18h, when phenotypic symptoms were not yet visible, only 154 genes exhibited differential expression compared to uninfected plants (153 up-regulated and 1 down-regulated). Interestingly, 588 out of 1299 genes induced by OGs treatment in seedlings after either 1 h or 3 h were

also up-regulated by *B. cinerea* infection at either time point analysed. Similarly, 316 out of 577 genes that were significantly repressed by OGs at either 1 h or 3 h were also down-regulated during fungal infection. Specifically, P450s involved in the biosynthesis of aliphatic glucosinolates like *CYP79F1* and *CYP83A1* were down regulated by both OGs and *B. cinerea*. In contrast, genes encoding P450s involved in the biosynthesis of indolic compounds like *CYP79B2*, and *CYP71B15* were up-regulated by both OGs and *B. cinerea*. Furthermore, *CYP81F2,* but not *CYP82C3,* was induced by both OGs and *B. cinerea*. This analysis indicates that about half of the genes responsive to OGs are also responsive in the same direction upon *B. cinerea* infection.

Chapitre 1; Paper 2

Ferrari et al., 2007

Resistance to Botrytis cinerea Induced in Arabidopsis by Elicitors Is Independent of Salicylic Acid, Ethylene, or Jasmonate Signaling But Requires PHYTOALEXIN DEFICIENT3

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Plant Physiology, 2007, vol. 144, pages 367-379

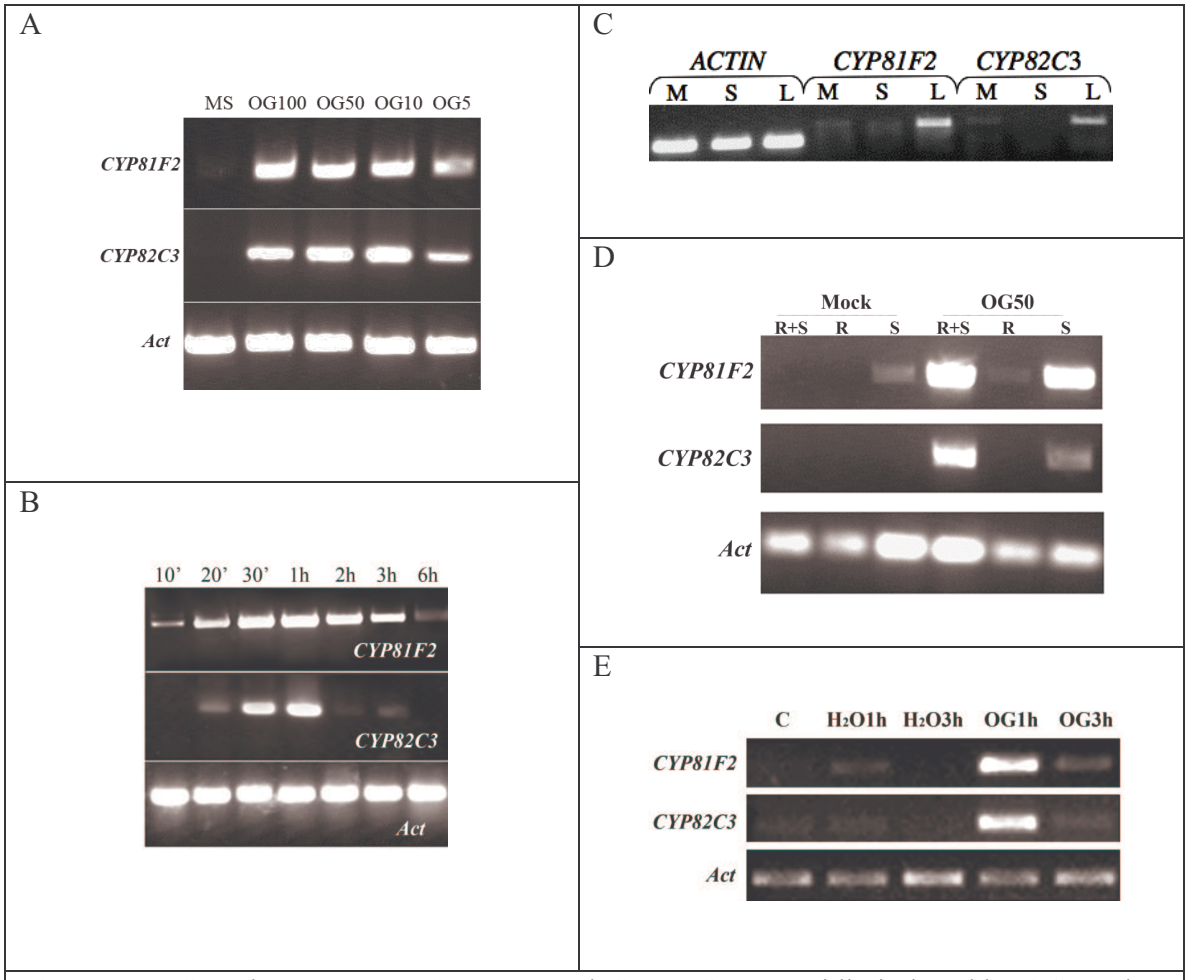


Figure 1-2: Cytochromes P450 *CYP81F2* and *CYP82C3* are rapidly induced by OGs and are predominantly expressed in shoot tissue.

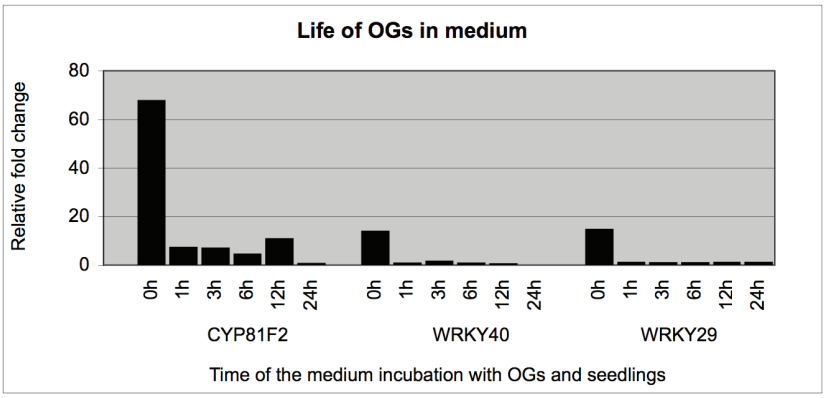
A. Dose/response of gene induction in seedlings treated with different concentrations (as indicated) of OGs for 1h
B. Kinetics of *CYP81F2* and *CYP82C3* induction in seedlings treated with 50 µg/ml OGs.
C. Response of marker genes in seedlings treated for 1h with different polymers: Oligomannuronide (M); Short OG (S), under 3 oligomers; Long OGs (L), 10-16 oligomers.
D. Tissue specificity of *CYP81F2* and *CYP82C3* expression after 1h treatment with 50µg/ml OGs: gene expression in the Roots (R), in the Shoots (S) or in the entire seedling (R+S).
E. OGs response in adult plant after 1h and 3h treatment of 200 □g/ml.
At the exception of the polymers experiment in panel E, the Oligogalacturonides (OGs) used were with a degree of polymerisation (DP) between 10-16.

5- A specific response to OGs DP 10-15, is responsible for early gene expression in seedlings

The transcriptional analysis carried out on Arabidopsis seedlings using Affymetrix ATH1 GeneChips suggested that the response to OGs is strong around 1h but declines rapidly when either 200µg/ml (in the 1h and 6h OGs transcript profiling experiment) or 50µg/ml (in the 1 and 3h OGs transcript profiling experiment) of OGs were used in the seedling assay. To ensure that a saturating concentration of OGs was being used, a kinetic and a titration assay were performed using *CYP81F2* and *CYP82C3* as marker genes of the early OG response. One hour of treatment was used for the titration experiment because *CYP81F2* and *CYP82C3* were highly induced at this time point based on the microarray data. In both cases a diminution of expression in response to OGs was only observed at 5µg/ml (Figure 1-2A). However, not all the genes differentially expressed in response to OG treatment are induced as strongly as the two P450s. Based on these data, a concentration of 50µg/ml of OGs was used for kinetic assays and further experiments, a dose that had also previously been shown to induce the maximal increase in cytosolic Ca^{2+} and H_2O_2 (Hu et al., 2004). The kinetic analysis (Figure 1-2B) showed that *CYP81F2* and *CYP82C3* are transiently up-regulated within 30 minutes of OGs treatment, with a peak in transcript levels between 30 min and 1 hour. *CYP81F2* induction in response to OGs is sustained over a longer time compared to *CYP82C3*.

Whether changes in gene expression upon treatment with OGs were due to specific perception of a particular length of oligogalacturonide and not the consequence of non-

A



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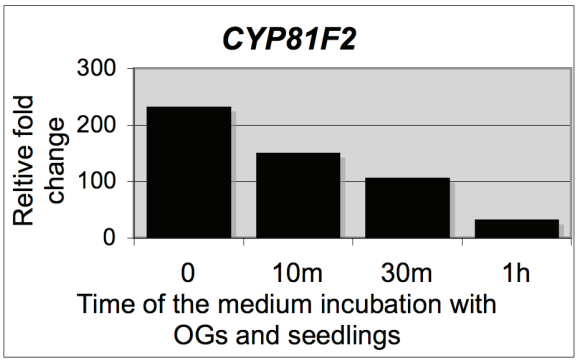

Figure 1-3: OGs activity in liquid media that contains seedlings.

A. One, 3, 6, 12, or 24 hours after addition of 50 µg/ml OGs to medium containing seedlings, the medium (conditioned medium) was removed and added to fresh seedlings. The fresh seedlings were subsequently assayed for response 1h after addition of the conditioned medium.
B. Ten, 30 minutes and 1 hour after addition of 50 µg/ml OGs to medium containing seedlings, the medium (conditioned medium) was removed and added to fresh seedlings. The fresh seedlings were subsequently assayed for response 1h after addition of the conditioned medium.

specific sensing of sugar polymers was investigated. Arabidopsis was treated with molecules structurally related to OGs. Seedlings were incubated for 1 h in the presence of 50 μg/ml short OGs (S), an oligomer of galacturonic acid with a degree of polymerization (DP) of 3; 50 μg/ml long OGs (L) an oligomer of galacturonic acid with a DP between 10-15-mer; or 50 μg/ml oligomannuronides (M) an oligomer of mannuronic acid, which is an epimer of galacturonic acid. As shown in Figure 1-2C, *CYP81F2* and *CYP82C3* transcripts were not elicited after treatment with oligomannuronide or short OGs. These results suggest the size and conformation of oligogalacturonides are specifically recognized by the plant. Additionally, as shown in Figure 1-2D, the two P450s were expressed in shoots of seedlings but not in roots, suggesting that the OGs response is localized in the shoots of Arabidopsis seedlings.

6- OGs are only active for less than an hour in the seedling assay

The observation that the response to OGs was rapid and transient and returned to a basal level within a few hours raised several questions. Is the transient nature of the response an intrinsic characteristic of this endogenous elicitor, or is the attenuation of the response due to the degradation or inactivation of OGs in the medium? To address these questions, we first determined the life of the active OGs in the seedling system. Medium in which seedlings (S1) had been exposed to OGs from 1 to 24 hours (Figure 1-3A) or 10 minutes to 1 hour (Figure 1-3B) was removed and added to fresh seedlings (S2). The newly treated seedlings were assayed by quantitative RT-PCR for elicitor response 1h after the addition of the medium. The expression of three OGs-elicited genes were

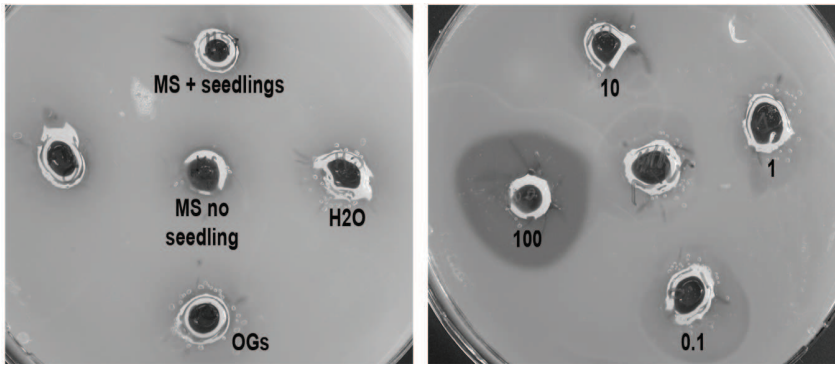

Figure 1-4: Polygalacturonases are not responsible for degradation of OGs in the medium.

On a 5% polygalacturonic acid (Fluka) substrate, 5 mm diameter holes were filled with 50 µl of different solutions.
Right panel: the holes were filled with solutions containing different concentrations of polygalacturonase indicated as µg/ml. Left panel: the holes were filled with MS medium (MS no seedlings), in which seedlings had been growing for 10 days (MS + seedlings), MS medium in which seedlings had been growing for 10 days and then treated with H_2O (H_2O) or 50 µg/ml OGs (OGs) for 1h.

monitored in these experiments, *CYP81F2, WRKY40 (At1g80840) and WRKY29 (At4G23550).* WRKY40 is a modulator of the SA and JA signaling pathways, functioning as an activator of JA-dependent defense pathways and a repressor of SA signaling (Dong et al., 2003; Xu et al., 2006). WRKY29 is involved in the resistance response to both bacterial and fungal pathogens (Asai et al., 2002). Because it was difficult to design specific primers for *CYP82C3* as this gene shares 80% or more identity with *CYP82C2* and *CYP82C4,* it was not included in these experiments.

The three reporter genes failed (Figure 1-3A) to respond to medium in which OGs had been pre-incubated for 1 hour with the seedlings (S1). The data in Figure 1-3B suggest that OGs activity is decreasing within 10 minutes in the presence of seedlings and is abolished after 1 hour.

To ascertain whether OGs are degraded by potential polygalacturonases released by the plant into the medium, we assayed whether polygalacturonase activity was present in the medium in which seedlings had been growing for 10 days or in medium in which 10-day old plants were incubated with 50µg/ml OGs for 1 hour. A 0.5 cm well was made in agarose containing 0.5% polygalacturonic acid and filled with 50 µl of medium or polygalacturonase solutions as a positive control. The plates were incubated 24 hours at 30°C and treated with 6N HCL. As shown in Figure 1-4, we could not detect any polygalacturonase activity in the medium from the seedlings whether they were treated with OGs or not. Halos were visible only around wells incubated with polygalacturonase solution, with a dose as low as 0.1 □g/ml. These data suggest that the disappearance of OGs from the medium is not caused by degradation of the OGs by polygalacturonases

released by the seedlings. It is possible that OGs are binding to plant cell wall components or are being inactivated in some other way.

7- Transcriptional response in adult plants

As described above, we showed with Simone Ferrari that, based on microarray data, the OG response in seedlings is correlated with the response to infection with *B. cinerea* in adult plants. This suggests that the response to OGs is probably similar in seedlings and in adult plants. To provide evidence to support this conclusion, we carried out semi-quantitative RT-PCR analysis of *CYP81F2* and *CYP82C3* expression in adult leaves infiltrated with 200µg/ml OGs. As in seedlings, both genes were transiently expressed at 1 hour after infiltration but had returned to basal levels by three hours after infiltration (Figure 1-2E). These data indicate that the transcriptional pattern of OG-elicited gene expression is similar in mature plants and in seedlings.

8- OGs protect seedlings from *P. syringae* infection

It has been reported that OG-treatment increases the resistance of several plants to various pathogens, including a 50% reduction of *B. cinerea* lesions on grapevine leaves (Aziz et al., 2004) and *Pseudomonas syringae* resistance in elicited tobacco (Baker et al., 1990). Simone Ferrari reported a local and systemic resistance to *B. cinerea* induced by OG treatment on Arabidopsis leaves (Ferrari et al., 2007). We could not perform a *B. cinerea* infection assay using the Arabidopsis seedling system because the fungus grows

well in MS medium and does not infect the seedlings. In contrast to the fungus, graduate student Wisuwat Songnuan in the Ausubel lab observed that *P. syringae* infects and multiplies in Arabidopsis seedlings growing in liquid but is not able to grow in MS liquid medium independently of seedlings. OGs were added to the seedlings at the same time as *P. syringae* DC3000. With an inoculum of OD_{600}=0.002, OGs were not able to limit the bacterial growth. Interestingly, when the bacterial inoculum was OD_{600}=0.0002, OGs limited the bacterial growth initially at an 18h time point, but eventually the bacteria grew to the same final titer after 48h, irrespective of whether the seedlings were treated with OGs (Figure 1-5). In any case, these data show that OGs are able to partially protect the seedling from *P. syringae* infection.

9- OG elicited responses are independent of SA, JA, and ET signaling

Salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) signaling pathways have been extensively studied, but their involvement in MAMP elicited signaling is poorly understood. Although there is ample data to suggest that the early gene activation elicited by MAMPs does not depend on SA, JA or ET, hormone elicited signaling does eventually appear to play an important role in defense gene expression. The multiplication of new pathways involved in plant defense signaling reflects the high complexity of plant mechanisms to prevent microorganism invasion. The marker genes *CYP81F2* and *CYP82C3* are expressed after 1 hour OGs elicitation (Figure 1-6) in Arabidopsis seedlings impaired in each classical defense signaling pathway: *npr1* is impaired in SA signaling, *ein2* is impaired in ethylene signaling, and *jar1* is impaired in

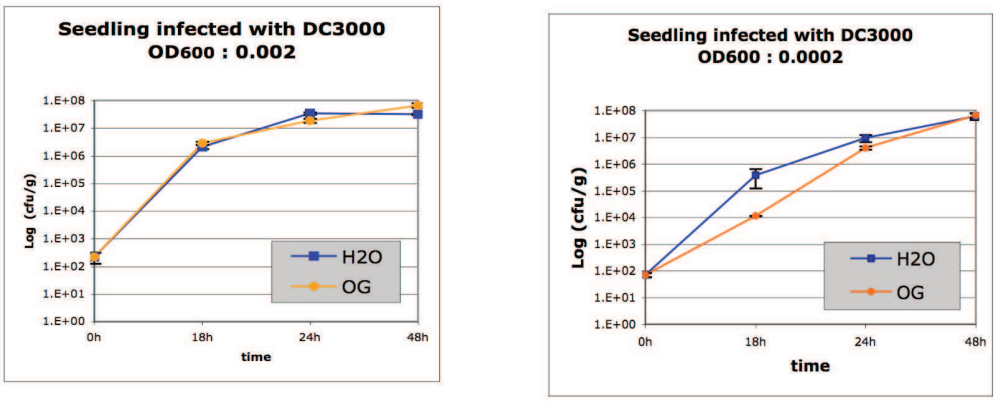

Figure 1-5: OGs induce transient protection against *Pseudomonas syringae* pv. *tomato* DC3000 in seedlings

Seedlings were inoculated with *Pseudomonas syringae* pv. *tomato* DC3000 at a concentration of OD600 = 0.002 or 0.0002 and harvested at times indicated for bacterial counts.

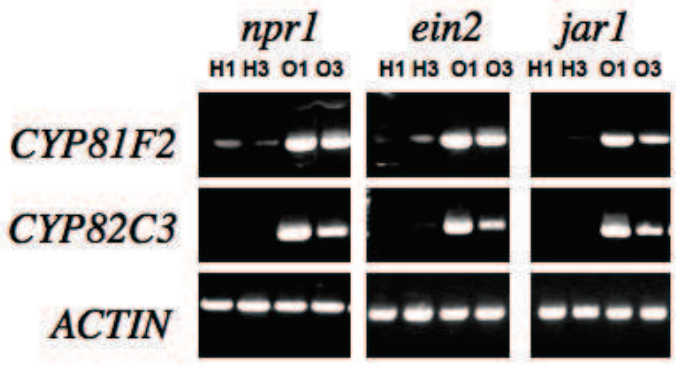

Figure 1-6: OGs induce an early response that is independent of SA, JA and Et signaling pathways.

Arabidopsis seedlings defective in SA (*npr1*), JA (*jar1*) and Et (*ein2*) signaling pathways were treated with 50ug/ml OGs and harvested after 1h.

JA responses. In addition, *CYP81F2*, *WRKY40*, and *AtPGIP1* are also induced by OGs in *eds16* impaired in SA signaling (Annex 1) (Chapter 1; Paper 3, supplemental data), (Galletti *et al*., 2008) and in a triple mutant, *npr1 ein2 jar1*, which is impaired in all three pathways (Chapter 1; Paper 3) (Galletti *et al*., 2008). Such data indicate that the early OGs response is mediated by (a) signaling pathway(s) that are independent of salicylic acid, jasmonic acid, or ethylene.

Chapitre 1; Paper 3

Galletti et al., 2008

The AtrbohD-Mediated Oxidative Burst Elicited by Oligogalacturonides in Arabidopsis Is Dispensable for the Activation of Defense Responses Effective against *Botrytis cinerea*[1][W][OA]

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Oligogalacturonides (OGs) are endogenous elicitors of defense responses released after partial degradation of pectin in the plant cell wall. We have previously shown that, in Arabidopsis (*Arabidopsis thaliana*), OGs induce the expression of *PHYTOALEXIN DEFICIENT3* (*PAD3*) and increase resistance to the necrotrophic fungal pathogen *Botrytis cinerea* independently of signaling pathways mediated by jasmonate, salicylic acid, and ethylene. Here, we illustrate that the rapid induction of the expression of a variety of genes by OGs is also independent of salicylic acid, ethylene, and jasmonate. OGs elicit a robust extracellular oxidative burst that is generated by the NADPH oxidase AtrbohD. This burst is not required for the expression of OG-responsive genes or for OG-induced resistance to *B. cinerea*, whereas callose accumulation requires a functional AtrbohD. OG-induced resistance to *B. cinerea* is also unaffected in *powdery mildew resistant4*, despite the fact that callose accumulation was almost abolished in this mutant. These results indicate that the OG-induced oxidative burst is not required for the activation of defense responses effective against *B. cinerea*, leaving open the question of the role of reactive oxygen species in elicitor-mediated defense.

Plants need to recognize invading pathogens in a timely manner to mount appropriate defense responses. Specific molecules associated with different microbial pathogens can be perceived by plant cells at early stages of infection and trigger inducible defenses that include phytoalexin accumulation, expression of pathogenesis-related proteins, production of reactive oxygen species (ROS), and, at least in some cases, programmed cell death. Many of these molecules, traditionally called general elicitors, are secreted or are present on the surface of all strains of a given microbial taxonomic group and activate defense responses effective against a wide range of pathogens (Nurnberger et al., 2004). For this reason, they are also referred to as microbe-associated molecular patterns or pathogen-associated molecular patterns (PAMPs; Parker, 2003; He et al., 2007). PAMPs (for review, see Nurnberger and Brunner, 2002) are often structural components of the pathogen cell wall (e.g. chitin, glucan) or other macromolecular structures (e.g. bacterial flagellin).

Hahn and colleagues (1981) first showed that structural components of the plant cell wall, released during pathogen infection as a consequence of microbial enzymatic activities, can also induce defense responses. In particular, oligogalacturonides (OGs) with a degree of polymerization (DP) between 10 and 15 can accumulate when fungal polygalacturonases (PGs) degrade the homogalacturonan component of plant pectin (Hahn et al., 1981). OGs elicit a variety of defense responses, including accumulation of phytoalexins (Davis et al., 1986), glucanase, and chitinase (Davis and Hahlbrock, 1987; Broekaert and Pneumas, 1988). Exogenous treatment with OGs protects grapevine (*Vitis vinifera*) and Arabidopsis (*Arabidopsis thaliana*) leaves against infection with the necrotrophic fungus *Botrytis cinerea* (Aziz et al., 2004; Ferrari et al., 2007), suggesting that production of this elicitor at the site of infection, where large amounts of PGs are secreted by the fungus, may contribute to activate defenses responses. For these reasons, OGs can be considered as danger

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signals derived from an altered self (host-associated molecular patterns).

A prominent feature of the plant defense response is the oxidative burst, a common early response of plant cells to pathogen attack and elicitor treatment (Lamb and Dixon, 1997). ROS such as superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) are toxic intermediates resulting from reduction of molecular O_2. ROS are important signals for defense responses and phytoalexin accumulation in several species. It is generally thought that ROS contribute to plant resistance by directly exerting a cytotoxic effect against pathogens, by participating in cell wall reinforcement (cross-linking of structural protein and lignin polymers), or by inducing hypersensitive cell death, expression of defense genes, or the accumulation of antimicrobial compounds (Levine et al., 1994). Generation of ROS can be induced by a variety of elicitors (Apostol et al., 1989; Legendre et al., 1993; Bolwell et al., 2002; Aziz et al., 2003; Kasparovsky et al., 2004; Pauw et al., 2004; Xu et al., 2005) and in many plant systems ROS production is biphasic (e.g. Dorey et al., 1999; Yoshioka et al., 2001).

O_2^--generating NADPH oxidases are generally considered to be a major enzymatic source of ROS in the oxidative burst of plant cells challenged with pathogens or elicitors (Torres and Dangl, 2005; Torres et al., 2006). Two different NADPH oxidase genes in potato (*Solanum tuberosum*) are responsible for the elicitor-induced biphasic oxidative burst (Yoshioka et al., 2001). In Arabidopsis, several genes encoding proteins with high similarity to the mammalian NADPH oxidase $gp91^{phox}$ subunit have been characterized. Among them, *AtrbohD* is required for the production of ROS during infection with different bacterial and fungal pathogens, including *B. cinerea* (Torres et al., 2002, 2005). Besides NADPH oxidases, other enzymes appear to be important in the elicitor-mediated oxidative burst, including apoplastic oxidases, such as oxalate oxidase (Dumas et al., 1993), amine oxidase (Allan and Fluhr, 1997), and pH-dependent apoplastic peroxidases (Bolwell et al., 1995; Frahry and Schopfer, 1998), which generate either O_2^- or H_2O_2.

We have recently shown that OGs and an unrelated elicitor, the synthetic 22-amino acid peptide flg22 derived from bacterial flagellin (Felix et al., 1999), activate defense responses against *B. cinerea* both in wild-type Arabidopsis and in mutants impaired in salicylic acid (SA), jasmonate (JA)-, or ethylene (ET)-mediated signaling (Ferrari et al., 2007). Elicitor-induced protection against *B. cinerea* requires the *PHYTOALEXIN DEFICIENT3* (*PAD3*) gene (Ferrari et al., 2007). *PAD3* encodes the cytochrome P450 CYP71B15, which catalyzes the last step of the biosynthesis of the phytoalexin camalexin (Schuhegger et al., 2006). Camalexin is known to contribute to Arabidopsis basal resistance to *B. cinerea* (Ferrari et al., 2003a; Kliebenstein et al., 2005). Notably, the expression of *PAD3*, as well as that of another defense-related gene, *AtPGIP1*, which encodes a PG-inhibiting protein effective against *B. cinerea*, is induced by OGs independently of SA-, JA-, and ET-mediated signaling (Ferrari et al., 2003b, 2007). It is therefore likely that multiple defense responses are induced by OGs independently of SA, ET, and JA.

Transient accumulation of extracellular H_2O_2 was previously observed in tobacco (*Nicotiana tabacum*) leaf explants and grapevine cells treated with OGs (Bellincampi et al., 1996; Aziz et al., 2004). Because *PAD3* expression and camalexin accumulation can be induced by chemicals that generate oxidative stress (Zhao et al., 1998; Denby et al., 2005), we have investigated the hypothesis that H_2O_2 mediates the induction of defense responses effective against *B. cinerea* in Arabidopsis plants treated with OGs. Here, we show that OGs induce an oxidative burst in Arabidopsis that is AtrbohD-dependent; however, we also show that H_2O_2-dependent responses are not required for OG-induced resistance against *B. cinerea*.

RESULTS

Early Activation of Genes in Response to General Elicitors Is Independent of SA, ET, and JA Signaling

To establish the degree of specificity of early gene expression in response to OGs and other general elicitors, we monitored the expression of *AtPGIP1*, *PAD3*, and several other early elicitor-induced genes (Ferrari et al., 2007; Denoux et al., 2008) in response to a pool of OGs with a DP between 10 and 15 (hereafter referred to as OGs), to purified oligodecagalacturonic acid (DP10), to flg22, and to a β-glucan elicitor from *Phytophthora megasperma* f. sp. *Glya* (Cheong et al., 1991). In addition to *AtPGIP1* and *PAD3*, we tested the expression of *AtWRKY40* (At1g80840), encoding a transcription factor that acts as a negative regulator of basal defense (Xu et al., 2006; Shen et al., 2007); *CYP81F2* (At5g57220), encoding a cytochrome P450 with unknown function; and *RetOx* (At1g26380), encoding a protein with homology to reticuline oxidases, a class of enzymes involved in secondary metabolism and in defense against pathogens (Dittrich and Kutchan, 1991; Carter and Thornburg, 2004). These genes were selected because they are rapidly and strongly up-regulated upon exposure to elicitors, as previously demonstrated by whole-genome transcript profiling and real-time quantitative PCR analyses (Ferrari et al., 2007; Denoux et al., 2008). As negative controls, we treated seedlings with water or α-1,4-trigalacturonic acid (DP3; Hahn et al., 1981; Cervone et al., 1989; Bellincampi et al., 2000; Navazio et al., 2002).

As shown in Figure 1, OGs, DP10, flg22, and β-glucan activated the expression of all tested genes in Arabidopsis seedlings, whereas water and DP3 failed to induce the expression of any of the genes analyzed. The expression of *PAD3*, *RetOx*, *CYP81F2*, *AtWRKY40*, and *AtPGIP1* was also compared across a set of 322 publicly available Arabidopsis microarray datasets using the Arabidopsis Coexpression Tool (Manfield

et al., 2006). The Pearson correlation coefficient between *PAD3* and *RetOx* expression was the highest (r = 0.78) among the tested genes (Supplemental Fig. S1), followed by *RetOx* and *CYP81F2* (r = 0.71). The *AtWRKY40* expression pattern appeared to correlate moderately with that of *PAD3* and *RetOx* (r = 0.58 in both cases), whereas no significant correlation between *AtPGIP1* and any of the other genes was observed, suggesting that the expression of this gene is regulated differently from that of *PAD3*, *RetOx*, and *CYP81F2*. Despite the fact that *AtPGIP1* does not significantly correlate with any other analyzed gene, it was included in subsequent analyses because of its established role in plant defense (Ferrari et al., 2003b, 2006). Transient expression of *PAD3*, *RetOx*, *CYP81F2*, and *AtWRKY40* was also observed in rosette leaves infiltrated with OGs (Supplemental Fig. S2) with kinetics comparable to those occurring in seedlings, indicating that these genes can be considered markers of early elicitor-induced responses both in seedlings and in adult plants.

To determine whether *RetOx*, *CYP81F2*, and *AtWRKY40* are expressed after elicitor treatment independently of SA, ET, or JA, as previously shown for *AtPGIP1* and *PAD3* (Ferrari et al., 2003b, 2007), we analyzed their expression in the *npr1ein2jar1* (*nej*) genetic background harboring mutations in the *NON-PR1 EXPRESSOR1* (*NPR1*; Cao et al., 1997), *JASMONATE RESISTANT1* (*JAR1*; Staswick et al., 1992), and *ETHYLENE INSENSITIVE2* (*EIN2*; Guzman and Ecker, 1990) genes, and therefore impaired in all three signaling pathways (Clarke et al., 2000). No major difference in expression of *RetOx*, *CYP81F2*, and *AtWRKY40* was observed, either in terms of kinetics of induction or in transcript levels, in wild-type or *nej* plants treated with OGs (Fig. 2, A–C), or in *npr1*, *ein2*, and *jar1* single mutants (Supplemental Fig. S3A). Expression of *AtPGIP1*, that was previously shown to be independent of *JAR1*, *EIN2* or *NPR1*, based on data obtained with single mutants (Ferrari et al., 2003b), was also unaffected in the triple mutant (Fig. 2D).

Because some reports have suggested that the *jar1-1* mutation is leaky (Staswick et al., 1998; Kariola et al.,

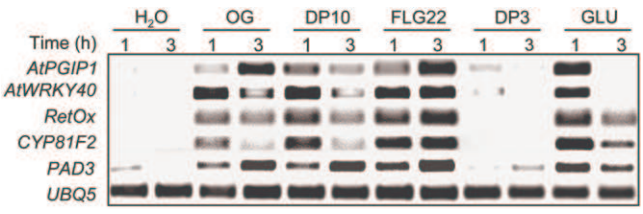

Figure 1. Expression analysis of marker genes in response to elicitors. Arabidopsis seedlings were treated at the indicated time (h) with water (H_2O), OGs, purified oligodecagalacturonic acid (DP10), flg22, trigalacturonic acid (DP3), or β-glucan (GLU). Expression of the indicated genes was analyzed by semiquantitative RT-PCR, using the *UBQ5* gene as internal standard. This experiment was repeated twice with similar results.

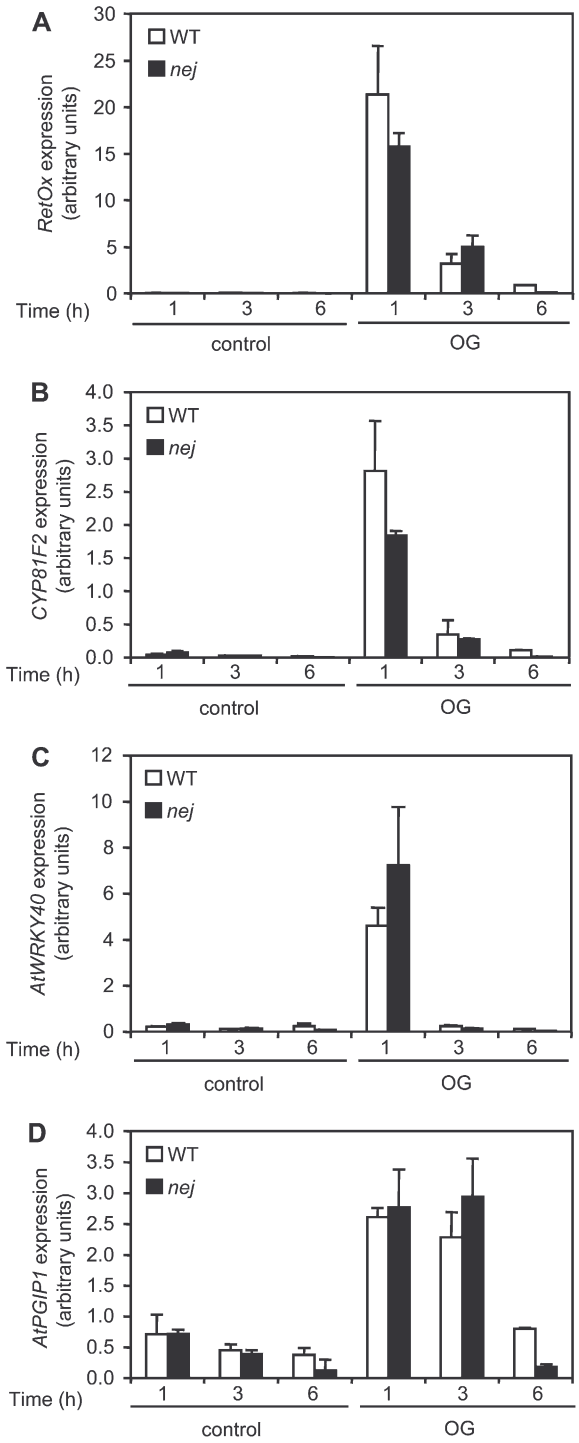

Figure 2. Expression of elicitor-responsive genes in the *nej* triple mutant. Arabidopsis wild-type (white bars) or *nej* triple mutant (black bars) seedlings were treated with water (control) or OGs for 1, 3, or 6 h. Expression of *RetOx* (A), *CYP81F2* (B), *AtWRKY40* (C), and *AtPGIP1* (D) was analyzed by real-time quantitative PCR and normalized using the expression of the *UBQ5* gene. Bars indicate average expression ± SD of three replicates. This experiment was repeated three times with similar results.

2003), we also analyzed the *coronatine insensitive1* (*coi1*) mutant, which is severely impaired in JA-mediated responses (Xie et al., 1998). Induction of *RetOx* and *CYP81F2* by OGs in wild-type and *coi1* seedlings was indistinguishable, whereas *AtWRKY40* expression was slightly reduced in *coi1* (Supplemental Fig. S3B), in accordance with a previous report indicating that *AtWRKY40* gene can be induced by JA in a *COI1*-dependent manner (Wang et al., 2008). Similarly, to further rule out an effect of SA on OG-induced gene expression, we treated *sid2-2* seedlings, which carry a mutation in the isochorismate synthase gene *ICS1* required for pathogen-activated biosynthesis of SA (Wildermuth et al., 2001). Also, in this case, no significant reduction of OG-induced gene expression was observed compared to the wild type (Supplemental Fig. S3C). These results indicate that expression of the OG-induced marker genes tested is independent of SA, ET, and JA.

Production of H_2O_2, But Not Gene Expression, in Response to OGs Is Mediated by AtrbohD

Analysis of the publicly available expression data using Genevestigator (https://www.genevestigator.ethz.ch) indicates that *PAD3*, *RetOx*, *AtWRKY40*, and *CYP81F2* transcript levels increase after treatment with H_2O_2, suggesting that their expression may be mediated by ROS (data not shown). Transient accumulation of extracellular H_2O_2 was previously observed in tobacco leaf explants and grapevine cells treated with OGs (Bellincampi et al., 1996; Aziz et al., 2004). To investigate whether OGs are also able to induce an apoplastic oxidative burst in Arabidopsis, we measured the release of H_2O_2 in the culture medium of seedlings treated with these elicitors. A significant oxidative burst was observed in response to OGs and DP10, whereas H_2O_2 accumulated to a much smaller extent in response to flg22, β-glucan, or DP3 (Fig. 3A).

We then investigated the source of H_2O_2 generated after treatment with OGs. Previous reports suggest that the oxidative burst observed after inoculation with virulent and avirulent pathogens is generated in Arabidopsis by the NADPH oxidase AtrbohD (Torres et al., 2005). To determine whether this enzyme is also the source of the extracellular burst observed in response to OGs, we analyzed an Arabidopsis knockout (KO) line containing a T-DNA insertion in the *AtrbohD* gene (Torres et al., 2002). This line failed to accumulate extracellular H_2O_2 after elicitation (Fig. 4A), indicating that AtrbohD is necessary for the OG-induced oxidative burst.

To determine the role of the oxidative burst in OG-triggered early gene expression, we analyzed the expression of *PAD3*, *RetOx*, *CYP81F2*, and *AtWRKY40* in elicited wild-type and *atrbohD* mutant seedlings. Strikingly, despite the absence of a functional *AtrbohD* gene and of an oxidative burst, no significant differences in the mRNA levels of all tested marker genes could be detected (Fig. 5). Similar results were obtained in wild-type and *atrbohD* adult plants infiltrated with OGs (Supplemental Fig. S2). To conclusively rule out a role of NADPH oxidases in OG-induced marker gene expression, before application of OGs, we treated seedlings with diphenylene iodonium (DPI), which, at low concentrations, specifically inhibits this class of enzymes (Bolwell et al., 1995; Frahry and Schopfer, 1998). DPI completely blocked the OG-induced oxidative burst (Fig. 6A), but had no effect on the expression of *PAD3*, *RetOx*, *CYP81F2*, and *AtWRKY40* (Fig. 6B), confirming that NADPH oxidases are not required for early OG-induced transcriptional changes.

To conclusively demonstrate that extracellular H_2O_2 is not involved in OG-induced gene expression, we elicited Arabidopsis seedlings in the presence of catalase at a concentration that almost completely abolished the oxidative burst (Fig. 3A). Coincubation of

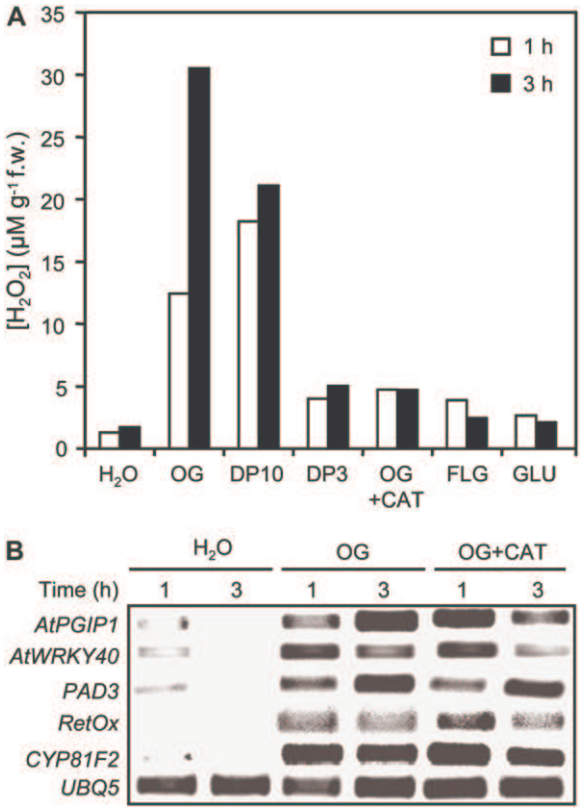

Figure 3. Oxidative burst and gene expression in response to elicitors. A, Arabidopsis seedlings were treated with water (H_2O), OGs alone, or in the presence of catalase (OG + CAT), purified oligodecagalacturonic acid (DP10), flg22 (FLG), trigalacturonic acid (DP3), or β-glucan (GLU). H_2O_2 accumulation in the culture medium, expressed as μM g^{-1} fresh weight, was measured after 1 (white bars) or 3 h (black bars). This experiment was repeated twice with similar results. B, Arabidopsis seedlings were treated for 1 or 3 h with water (H_2O) or with OGs alone or in presence of catalase (OG + CAT). Expression of the indicated genes was analyzed by semiquantitative RT-PCR, using the *UBQ5* gene as internal standard. This experiment was repeated twice with similar results.

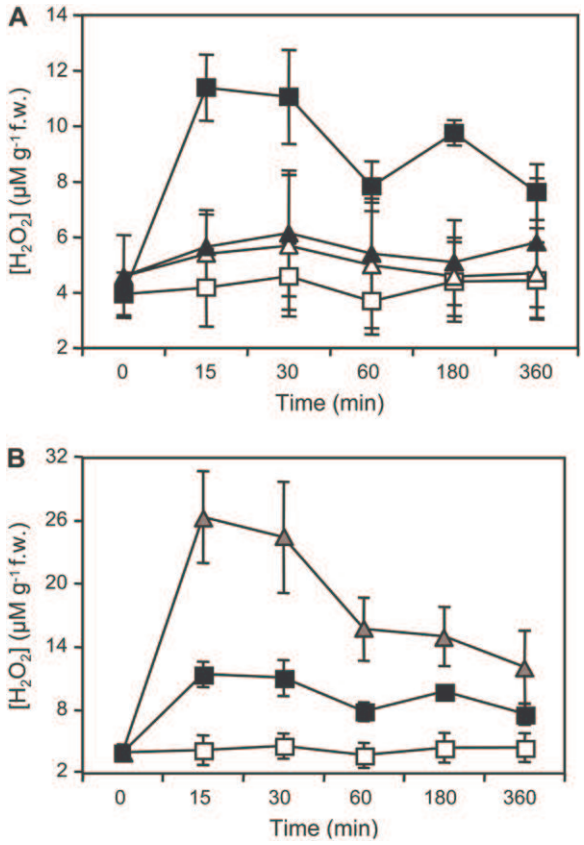

Figure 4. Accumulation of extracellular H_2O_2 in response to OGs or G/GO in Arabidopsis seedlings. A, Arabidopsis wild-type and *atrbohD* seedlings were treated with water (H_2O) or OGs for the indicated time (min). Arabidopsis wild-type (squares) and *atrbohD* (triangles) seedlings were treated with water (white symbols) or OGs (black symbols). B, Arabidopsis seedlings were treated with water (H_2O, white squares), OGs (black squares), or G/GO (gray triangles). H_2O_2 accumulation in the culture medium, expressed as μM g^{-1} fresh weight, was measured at the indicated times (min). Values are means of three samples ± SD.

OGs with catalase had no significant effect on the expression of *PAD3*, *AtPGIP1*, *RetOx*, *CYP81F2*, and *AtWRKY40* (Fig. 3B), confirming that H_2O_2 is not required for OG-induced marker gene expression. Furthermore, treatment of seedlings with Glc and Glc oxidase (G/GO) at concentrations that induced H_2O_2 levels in the same order of magnitude observed after OG treatments (Fig. 4B), failed to induce the expression of the same set of genes (Fig. 5).

Taken together, our results indicate that OG-mediated early gene expression is independent of the extracellular oxidative burst.

Basal and OG-Induced Resistance to *B. cinerea* Infection Are Independent of AtrbohD and of PMR4/GSL5

To determine whether defense responses that occur relatively late after treatment with OGs are also independent of H_2O_2, we analyzed callose deposition and induced resistance in wild-type and *atrbohD* KO plants. Callose is a high-M_r β-1,3-glucan deposited at the site of infection by pathogens, probably acting as a physical barrier against colonization of the intercellular space (Ryals et al., 1996; Donofrio and Delaney, 2001). It was previously shown that flg22 induces callose deposition in Arabidopsis seedlings (Gomez-Gomez et al., 1999) and that callose accumulation induced by flg22 is impaired in leaf strips of *atrbohD* KO plants (Zhang et al., 2007). Similarly, infiltration of OGs in wild-type rosette leaves resulted in a significant accumulation of callose (Denoux et al., 2008), which was reduced of about 50% in *atrbohD* leaves (Fig. 7A), indicating that the oxidative burst contributes to callose synthesis also in response to OGs. As expected, infiltration of leaves of the *powdery mildew resistant4* (*pmr4*) mutant, which has a mutation in the callose synthase gene *GLUCAN SYNTHASE-LIKE5* (*GSL5*; Nishimura et al., 2003), resulted in a dramatic decrease of callose deposition (Fig. 7B).

We have previously observed that OGs induce protection of Arabidopsis plants against *B. cinerea* and that this protection requires *PAD3* expression (Ferrari et al., 2007). To determine the role of *AtrbohD* in induced resistance, we treated wild-type, *atrbohD*, and, as a negative control, *pad3* plants with OGs, and subsequently inoculated them with *B. cinerea*. As expected, *pad3* plants showed increased basal susceptibility, and OG pretreatment did not reduce lesion development (Fig. 8A). In contrast, no significant difference in basal susceptibility and in OG-induced resistance between wild-type and *atrbohD* plants was observed either in detached leaves (Fig. 8A) or in intact plants (Fig. 9). This indicates that OG-induced activation of defense responses effective against *B. cinerea* does not require *AtrbohD*.

Furthermore, we investigated the role of callose in OG-elicited resistance to *B. cinerea*. As shown in Figure 8B, lesion development in *pmr4* plants inoculated with *B. cinerea* was unaffected or, in some experiments, slightly reduced, compared to wild-type plants. Moreover, OG treatment of the *pmr4* mutant resulted in protection against *B. cinerea* infection (Fig. 8B), indicating that callose does not play a major role in either basal or elicitor-induced resistance against this pathogen.

Finally, we infiltrated adult rosette leaves with G/GO at concentrations that in seedlings induced production of H_2O_2 levels in the same order of magnitude observed after OG treatments. G/GO caused significant accumulation of H_2O_2 in infiltrated tissues (Fig. 10A), but did not alter basal resistance to *B. cinerea* (Fig. 10B). These data indicate that a moderate extracellular oxidative burst, comparable to that observed after OG treatment, is not sufficient to induce defense responses effective against *B. cinerea*.

DISCUSSION

One of the earliest responses observed in plants inoculated with a pathogen or treated with an elicitor

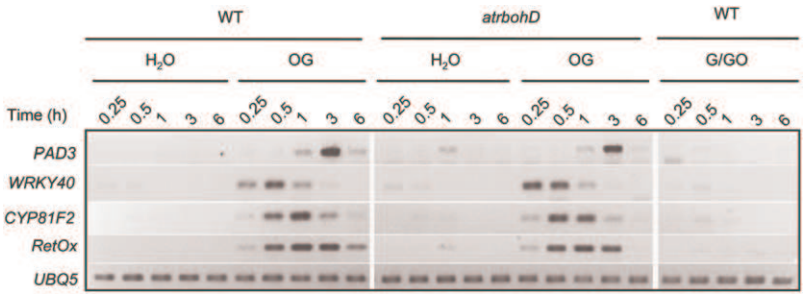

Figure 5. Effects of endogenous and exogenous H_2O_2 on OG-responsive genes. Arabidopsis wild-type and *atrbohD* seedlings were treated with water (H_2O) or OGs for the indicated time (h). Wild-type seedlings were also treated with G/GO. Expression of the indicated genes was analyzed by semiquantitative RT-PCR, using the *UBQ5* gene as internal standard. This experiment was repeated twice with similar results.

is the oxidative burst, characterized by a rapid and transient production of ROS. OGs induce a strong extracellular oxidative burst, initially suggesting that ROS might play an important role in mediating responses to OGs. We therefore adopted both pharmacological and genetic approaches to investigate both the genesis and the role of the oxidative burst elicited by OGs in Arabidopsis plants.

There are a number of potential sources of ROS generated upon pathogen or elicitor perception. Increasing evidence points to superoxide-generating NADPH oxidases as the main sources of extracellular ROS produced during pathogen infection or elicitation (Yoshioka et al., 2001, 2003; Torres et al., 2002; Kobayashi et al., 2006; Nuhse et al., 2007). O_2^- generated by NADPH oxidases is rapidly dismutated into H_2O_2, which is much more stable and can accumulate in tissues. Extracellular H_2O_2 can also be generated by other sources, most notably apoplastic peroxidases (Bolwell et al., 2002), making it sometimes difficult to discern the involvement of specific sources of ROS in the oxidative burst. The data presented here clearly indicate that the NADPH oxidase AtrbohD is necessary for the extracellular burst induced in Arabidopsis by OGs, as previously shown for flg22 (Nuhse et al., 2007). H_2O_2 produced after OG treatment is therefore likely released by dismutation of O_2^- directly generated by AtrbohD in accordance with the observation that OGs induce the accumulation of O_2^- in Arabidopsis leaves (Song et al., 2006). In addition to the extracellular oxidative burst, protoplastic sources of ROS emanating from mitochondrial, chloroplastic, or peroxisomal generating systems have also been documented (Bolwell et al., 2002). However, intracellular generation of ROS has mainly been studied in relation to abiotic stress (Asada, 1999; del Río et al., 2002). There are reports of intracellular accumulation of ROS in response to elicitors, such as cryptogein (Ashtamker et al. 2007), although its role in plant defense response has not been assessed.

OGs activate a very strong extracellular oxidative burst; surprisingly, however, this burst has a minor, if any, role in several downstream responses, based on the following evidence: (1) under our experimental conditions, there is significantly less H_2O_2 accumulation in response to flg22 and β-glucan than in response to OGs, but the effect of flg22 and β-glucan on the expression of early molecular marker genes is comparable to that observed with OGs; (2) H_2O_2 generated by G/GO at levels comparable to those observed in OG-treated plants fails to activate the expression of elicitor-activated marker genes or to induce resistance to *B. cinerea*; (3) scavenging of H_2O_2 accumulation by catalase or inhibition of the OG-induced oxidative burst either by DPI or by the *atrbohD* mutation did not affect

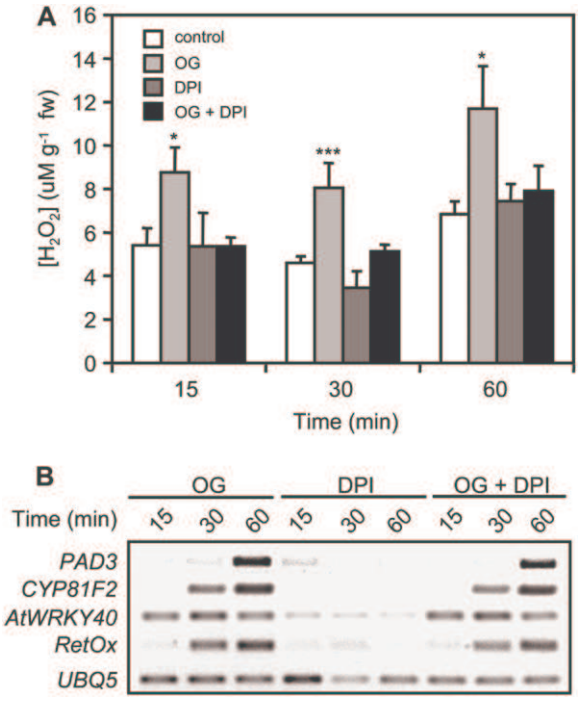

Figure 6. Effect of DPI on the expression of elicitor-responsive genes. A, Arabidopsis seedlings were treated with water (control, white bars), OGs (light gray bars), DPI (dark gray bars), or OGs + DPI (black bars). H_2O_2 accumulation in the culture medium, expressed as μM g^{-1} fresh weight, was measured at the indicated times (min). Values are means of three samples ± SD. Asterisks indicate statistically significant differences between control and OG-treated seedlings, according to Student's *t* test (*, $P < 0.05$; ***, $P < 0.01$). The experiment was repeated twice with similar results. B, Arabidopsis seedlings were treated with DPI, OGs alone, or in the presence of DPI (OG + DPI) for the indicated time (min). Gene expression was analyzed by semiquantitative RT-PCR, using the *UBQ5* gene as internal standard. This experiment was repeated twice with similar results.

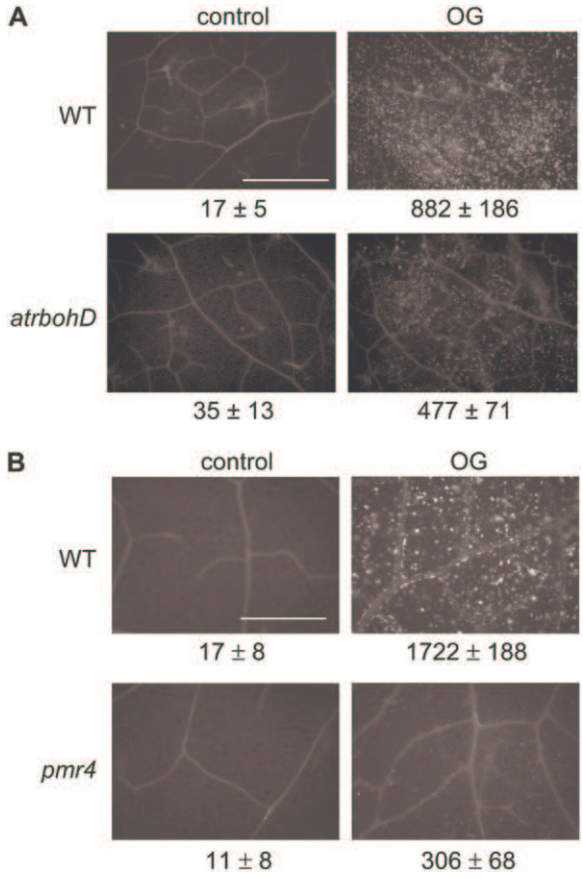

Figure 7. Callose accumulation in *atrbohD* and *pmr4* plants. Arabidopsis wild-type and *atrbohD* (A) or *pmr4* (B) leaves were infiltrated with water (control, left) or OGs (right) for 24 h and stained with aniline blue for callose visualization. The number below each image indicates the average number of callose deposits ± SE of eight different leaf samples from at least five independent plants (three microscopic fields of 0.1 mm^2 for each leaf). Images show representative leaves for each treatment. All images are at the same scale; scale bar = 1 mm (10× magnification). This experiment was repeated twice with similar results.

early gene expression. Taken together, these results indicate that early changes in gene expression activated by OGs independently of SA, ET, and JA do not require the oxidative burst generated by AtrbohD. Furthermore, OG-triggered resistance against *B. cinerea*, which is also independent of SA, ET, and JA, occurs in the absence of AtrbohD.

In contrast to OGs, flg22 and β-glucan elicited very low levels of H_2O_2 under our experimental conditions. An extracellular oxidative burst, peaking at about 10 to 15 min, was previously observed using a H_2O_2-dependent luminescence assay in Arabidopsis leaf explants treated with 1 μM flg22 (Gomez-Gomez et al., 1999). It is possible that the xylenol orange-based system used here is not sensitive enough to detect the burst induced by flg22, although previous work indicates the equivalence of this xylenol orange and the luminescence assays (Bindschedler et al., 2001). It is possible that the different levels of H_2O_2 that we observed after treatment with OGs or flg22 could be ascribed to different concentrations of the elicitors. However, at the doses used in this work, flg22 induced the expression of marker genes to levels comparable to OGs, indicating that the gene-activation response does not directly correlate to H_2O_2 accumulation. The observation that catalase, DPI treatments, or the *atrbohD* mutation block the oxidative burst, but have no significant impact on the expression of the early marker genes, confirms that the induction of these genes is uncoupled to ROS production.

The fact that none of the analyzed marker genes changed expression in response to H_2O_2 generated by G/GO was unexpected. Previous work showed that *PAD3* expression and camalexin accumulation can be up-regulated by ROS-generating chemicals (Zhao et al., 1998; Denby et al., 2005) and the expression of *CYP81F2*, *RetOx*, and *AtWRKY40* has been shown to be induced

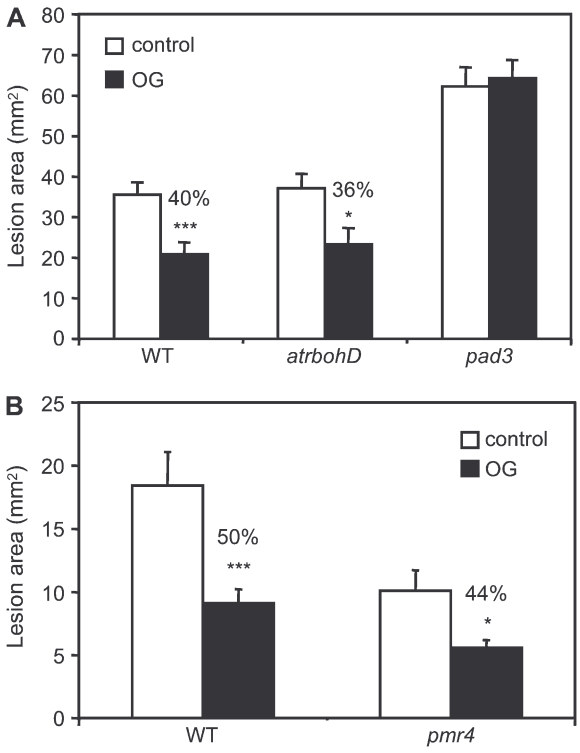

Figure 8. OG-induced resistance to *B. cinerea* is independent of AtrbohD and PMR4. A, Arabidopsis Col-0 (wild type), *atrbohD*, and *pad3* plants were treated with a control solution (white bars) or OGs (black bars) and inoculated with *B. cinerea* 24 h after treatment. B, Arabidopsis Col-0 (wild type) and *pmr4* plants were treated with a control solution (white bars) or OGs (black bars) and inoculated with *B. cinerea* 24 h after treatment. Lesion areas were measured 48 h after inoculation. Values are means ± SE of at least 14 lesions. Asterisks indicate statistically significant differences between control and OG-treated plants, according to Student's *t* test (*, $P < 0.05$; ***, $P < 0.01$). Numbers above bars represent the average reduction of lesion size (%) of OG-treated plants with respect to control-treated plants. The experiments were repeated at least twice with similar results.

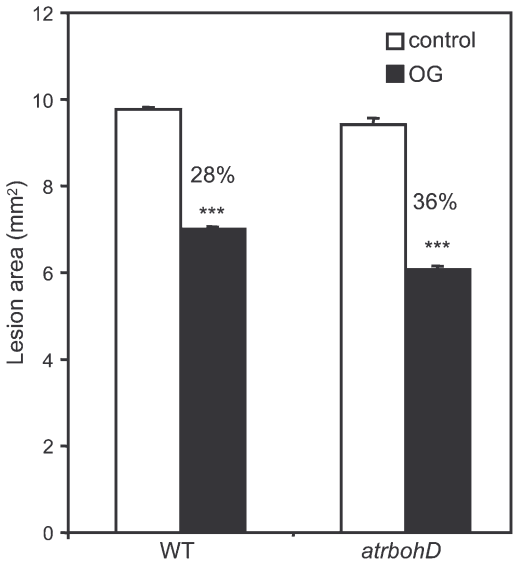

Figure 9. Basal and OG-induced resistance to *B. cinerea* in whole plants. Arabidopsis Col-0 (wild type) and *atrbohD* plants were treated with a control solution (white bars) or OGs (black bars) and leaves were inoculated with *B. cinerea* 24 h after treatment. Lesion areas were measured 48 h after inoculation. Values are means ± SE of at least 12 lesions. Asterisks indicate statistically significant differences between control and OG-treated plants, according to Student's *t* test (***, $P < 0.01$). Numbers above bars represent the average reduction of lesion size (%) of OG-treated plants with respect to control-treated plants. The experiments were repeated at least twice with similar results.

by millimolar concentrations of H_2O_2 (Davletova et al., 2005). However, the concentration of H_2O_2 measured in our experiments with G/GO was in the same order of magnitude as the concentration measured after elicitation with OGs (in the range of 10–30 μM g^{-1} fresh weight), which is comparable to the concentrations measured in leaves of different plant species under natural conditions (Cheeseman, 2006). This suggests that the relatively high concentrations of H_2O_2 used in previous expression analyses might be nonphysiological. Similarly, basal resistance to *B. cinerea* was not affected by treatment with G/GO at the same concentrations used in the seedling experiments. This result is apparently in contrast with a previous report indicating that G/GO infiltration of Arabidopsis leaves increases susceptibility to this pathogen (Govrin and Levine, 2000). However, the concentration of GO used by Govrin and Levine was 10^4-fold higher than in our work, suggesting that only very high levels of H_2O_2, which are not normally induced by elicitors, can affect basal resistance to *B. cinerea*.

Whereas OG-induced early gene expression and protection against *B. cinerea* occur independently of AtrbohD, callose accumulation is reduced in *atrbohD* KO plants. A similar result was obtained in *atrbohD* leaf strips treated with flg22 (Zhang et al., 2007). Callose deposition is required for β-amino butyric acid-induced resistance against the necrotrophic fungi *Alternaria brassicicola* and *Plectosphaerella cucumerina* (Ton and Mauch-Mani, 2004). Our observation that induced resistance to *B. cinerea* is unaffected in *atrbohD* plants, despite a reduction in callose accumulation, suggests that callose contributes only marginally to restrict *B. cinerea* in Arabidopsis. This hypothesis is confirmed by the observation that both basal and OG-induced resistance against *B. cinerea* are not impaired in the *pmr4* mutant, which accumulates very little callose.

Besides callose accumulation, other responses induced by OGs and other elicitors may be dependent on the oxidative burst. Previous reports suggest the existence of both oxidative burst-dependent and independent signaling pathways linking elicitor perception to downstream responses. Treatment of parsley (*Petroselinum crispum*) cells with DPI blocked both Pep-13-induced phytoalexin production and accumulation of transcripts encoding enzymes involved in their synthesis. In contrast, DPI had no effect on Pep-13-induced *PR* gene expression (Kroj et al., 2003). In grapevine, the expression of six out of nine defense-related genes responsive to OGs is blocked by DPI (Aziz et al., 2004), and in Arabidopsis Landsberg *erecta* seedlings treated with OGs, DPI blocks the expression of several defense genes (Hu et al., 2004). It is possible

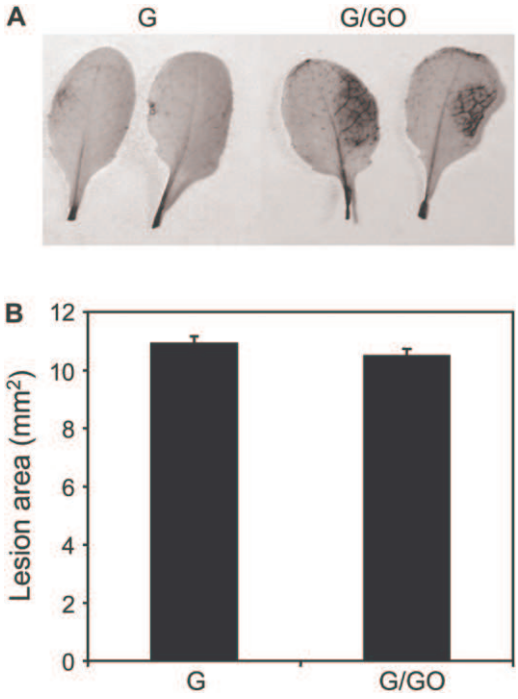

Figure 10. Basal resistance to *B. cinerea* after treatment with G/GO. A, Arabidopsis wild-type plants were infiltrated with Glc (G) alone or G/GO and, 24 h after treatment, stained with 3,3′-diaminobenzidine (DAB) for in vivo H_2O_2 visualization. B, Arabidopsis wild-type plants were infiltrated with Glc or G/GO and inoculated with *B. cinerea* 24 h after treatment. Lesion areas were measured 48 h after inoculation. Values are means ± SE of at least 12 lesions. No statistically significant differences between Glc- and G/GO-treated plants were observed, according to Student's *t* test ($P > 0.7$).

that the activation of a subset of late, secondary responses to elicitors is dependent, or at least is amplified by the earlier production of ROS.

CONCLUSION

In this work, we investigated the role of the extracellular oxidative burst in the induction of early and late responses to OGs in Arabidopsis plants. Our results indicate that OGs induce a transient, but robust, production of H_2O_2 that is dependent on the NADPH oxidase AtrbohD. This oxidative burst does not have a major role in the induction of several early OG-responsive marker genes and in the induced protection against *B. cinerea*. It was previously observed that early gene expression, in contrast to callose deposition, in response to the bacterial PAMP flg22, is independent of AtrbohD (Zhang et al., 2007). Here, we show that OGs, which are host-associated molecular patterns of a completely different chemical nature, behave in a similar fashion. However, we have demonstrated that defense responses that require the oxidative burst, such as callose deposition, are not involved in OG-induced resistance to *B. cinerea*. In contrast, flg22-induced resistance against *Pseudomonas syringae* infection is dependent on the NADPH oxidase AtrbohD (Zhang et al., 2007). Taken together, these results indicate that the signaling pathway activated by elicitors bifurcates: activation of one branch requires the oxidative burst and is important against bacterial pathogens, whereas the oxidative burst-independent branch regulates defense responses effective against necrotroph fungi.

MATERIALS AND METHODS

Plant Material

Arabidopsis (*Arabidopsis thaliana*) Columbia-0 (Col-0) wild-type seeds were purchased from Lehle Seeds. *pad3-1* (Glazebrook and Ausubel, 1994) and *eds16-1/sid2-2* (Wildermuth et al., 2001) mutant lines were previously described. Seeds of *ein2-1* and *jar1-1* were obtained from the Arabidopsis Biological Resource Center. The *npr1-1* line and the triple mutant *nej* were a kind gift from Xinnian Dong (Duke University). Heterozygous *coi1-1/COI1-1* seeds were a kind gift from John Turner (University of East Anglia). The *atrbohD* KO line was kindly provided by Jonathan G.D. Jones (Sainsbury Laboratory, John Innes Centre). The *pmr4-1* mutant line was kindly provided by Shauna C. Somerville (Carnegie Institution). All mutant lines used in this work are in the Col-0 background.

Growth Conditions and Plant Treatments

Plants were grown on a 3:1 mixture of soil (Einheitserde) and sand (Compo Agricoltura) at 22°C and 70% relative humidity under a 16-h light/8-h dark cycle (approximately 120 μmol m^{-2} s^{-1}). For OG treatments, leaves from 4-week-old plants were infiltrated with water or 200 μg mL^{-1} OGs using a needleless syringe and harvested at the indicated times. Generation of H_2O_2 in adult plants was obtained by infiltrating rosette leaves of 4-week-old plants with 0.25 mM Glc and 0.01 unit mL^{-1} Glc oxidase (Sigma). As a negative control, plants were infiltrated with 0.25 mM Glc alone.

For seedling treatments, seeds were surface sterilized and germinated in multiwell plates (approximately 10 seeds/well) containing 1 mL per well of Murashige and Skoog medium (Sigma; Murashige and Skoog, 1962) supplemented with 0.5% Suc. Plates were incubated at 22°C with a 12-h light/12-h dark cycle and a light intensity of 120 μmol m^{-2} s^{-1}. After 8 d, the medium was replaced and treatments were performed after two additional days. For treatment of *coi1* seedlings, heterozygous *coi1/COI1* seeds were first germinated on agar plates containing 30 μM methyl jasmonate, and, after 8 d of growth, homozygous JA-resistant seedlings were transferred to liquid Murashige and Skoog medium and treated with OGs 2 d later. As a control, wild-type seedlings were grown for 8 d on agar plates and then transferred to liquid Murashige and Skoog medium.

OG pools with an average DP of 10 to 15 (OGs) and purified decagalacturonic acid (DP 10) were kindly prepared by Gianni Salvi (Università di Roma "La Sapienza") as previously described (Bellincampi et al., 2000). Trigalacturonic acid (DP3) was purchased from Sigma. Matrix-assisted laser desorption/ionization time-of-flight MS was used to verify the DP of OG preparations. *Phytophthora megasperma* f. sp. *Glya* β-glucan elicitor was a kind gift of Michael G. Hahn (Complex Carbohydrate Research Center, University of Georgia). The flg22 peptide was synthesized by Maria Eugenia Schininà (Università di Roma "La Sapienza"). Lyophilized elicitors or chemicals were dissolved in double-distilled water and added to the culture medium at the following final concentrations: 100 μg mL^{-1} (approximately 54 μM) OG; 52 μg mL^{-1} (approximately 54 μM) DP10; 29 μg mL^{-1} (approximately 54 μM) DP3, 1 μM flg22 and 50 μg mL^{-1} β-glucan. H_2O_2 was removed from the culture medium by adding bovine catalase (Sigma) at the same time as OG at a final concentration of 600 units mL^{-1}. Generation of H_2O_2 was obtained by adding 0.25 mM Glc and 0.01 unit mL^{-1} Glc oxidase (Sigma) to the culture medium. DPI was prepared as a 1 mM stock in 20% dimethyl sulfoxide. DPI was added to the seedling growth medium at a final concentration of 10 μM, 15 min before OG treatment. As a control, dimethyl sulfoxide was added to the medium at a final concentration of 0.2%.

Botrytis cinerea growth and protection assays on detached leaves were performed as previously described (Ferrari et al., 2007). Infection of intact plants was performed by inoculating about three leaves per plant (at least four plants per genotype) with two 5-μL droplets of a *B. cinerea* spore suspension. Plants were subsequently covered with a plastic dome to keep humidity high as previously described (Ferrari et al., 2003a).

Determination of H_2O_2

The H_2O_2 concentration in the incubation medium of treated seedlings (about 100–120 mg in 1 mL of medium) was measured by the FOX1 method (Jiang et al., 1990), based on the peroxide-mediated oxidation of Fe^{2+}, followed by the reaction of Fe^{3+} with xylenol orange dye (*o*-cresolsulfonephthalein 3′,3′′-bis[methylimino] diacetic acid, sodium salt; Sigma). This method is extremely sensitive and used to measure low levels of water-soluble H_2O_2 present in the aqueous phase. To determine H_2O_2 concentration, 500 μL of the incubation medium were added to 500 μL of assay reagent (500 μM ammonium ferrous sulfate, 50 mM H_2SO_4, 200 μM xylenol orange, and 200 mM sorbitol). Absorbance of the Fe^{3+}-xylenol orange complex (A_{560}) was detected after 45 min of incubation. The specificity for H_2O_2 was tested by eliminating H_2O_2 in the reaction mixture with catalase. Standard curves of H_2O_2 were obtained for each independent experiment. Data were normalized and expressed as micromolar H_2O_2/g fresh weight of seedlings.

For in vivo H_2O_2 visualization, leaves were cut from infiltrated adult plants using a razor blade and dipped for 12 h in a solution containing 1 mg mL^{-1} of 3,3′-diaminobenzidine-HCl, pH 5.0. Chlorophyll was extracted for 10 min with boiling ethanol and for 2 h with ethanol at room temperature prior to photography (Orozco-Cardenas and Ryan, 1999).

Gene Expression Analysis

Treated seedlings or leaves were frozen in liquid nitrogen, homogenized with a mortar and pestle, and total RNA was extracted with Tri-Reagent (Sigma) according to the manufacturer's protocol. RNA was treated with RQ1 DNase (Promega) and first-strand cDNA was synthesized using ImProm-II reverse transcriptase (Promega) according to the manufacturer's instructions. Real-time quantitative PCR analysis was performed using an I-Cycler (Bio-Rad). Two microliters of a 1:5 dilution of cDNA (corresponding to 20 ng of total RNA) were amplified in a 30-μL reaction mix containing 1× IQ SYBR Green Supermix (Bio-Rad) and 0.4 μM of each primer. Expression levels of each gene, relative to *UBQ5*, were determined using a modification of the Pfaffl method (Pfaffl, 2001) as previously described (Ferrari et al., 2006). Semiquantitative reverse transcription (RT)-PCR analysis was performed in a

50-μL reaction mix containing 1 μL of cDNA, 1× buffer (Bioline), 3 mM $MgCl_2$, 100 μM of each dNTP, 0.5 μM of each specific primer, and 1 unit Taq DNA Polymerase (Bioline). Twenty-five, 30, and 35 PCR cycles were performed for each primer pair to verify linearity of the amplification. Primer sequences are shown in Supplemental Table S1. PCR products were separated by agarose gel electrophoresis and stained with ethidium bromide.

Pearson correlation coefficients between the expression pattern of selected genes in 322 Affymetrix ATH1 microarray datasets obtained from different Arabidopsis tissues and after different treatments and available in the Genomic Arabidopsis Resource Network/Nottingham Arabidopsis Stock Centre microarray database (Craigon et al., 2004) and scatter plots of the correlation coefficient values were obtained using the Arabidopsis Coexpression Tool (http://www.arabidopsis.leeds.ac.uk/act/index.php; Manfield et al., 2006). The scatter plot allows users to visualize the correlation of all probe sets against two selected probe sets simultaneously. Every probe set is plotted on a scatter graph, where the two axes are the Pearson correlation coefficients against two different query probe sets. Analysis of the expression of single genes in publicly available microarray experiments was performed using Genevestigator (https://www.genevestigator.ethz.ch; Zimmermann et al., 2004).

Callose Deposition

Leaves from 4-week-old plants were infiltrated with water or 200 μg mL^{-1} OGs using a needleless syringe. After 24 h, for each treatment, about eight leaves, from at least five independent plants, were cleared and dehydrated with 100% ethanol. Leaves were fixed in an acetic acid:ethanol (1:3) solution for 2 h, sequentially incubated for 15 min in 75% ethanol, in 50% ethanol, and in 150 mM phosphate buffer, pH 8.0, and then stained for 1 h at 25°C in 150 mM phosphate buffer, pH 8.0, containing 0.01% (w/v) aniline blue. After staining, leaves were mounted in 50% glycerol and examined by UV epifluorescence using an Axioskop 2 plus microscope (Zeiss). Images were taken with a ProgRes C10 3.3 MegaPixel digital color camera (Jenoptik). Callose quantification was performed by using ImageJ software.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Co-correlation between expression pattern of *PAD3* and *RetOx*.

Supplemental Figure S2. Expression of elicitor-responsive genes in adult Arabidopsis wild-type and *atrbohD* plants.

Supplemental Figure S3. Expression of selected marker genes in mutants impaired in SA, JA, and ET signaling.

Supplemental Table S1. Primers used in this article.

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DISCUSSION

Plant basal defense responses to pathogen attack involve recognition of molecules that characterize pathogens or molecules that are generated as the result of an infection of the plant tissue such as endogenous pectic cell wall fragments (oligogalacturonides: OGs). Oligogalacturonides mediate signaling, trigger defense responses and contribute to the early immune response like other well-studied MAMPs. Moreover, treatment with exogenous OGs enhances resistance against pathogens such as *B. cinerea* in grapevine and Arabidopsis (Aziz et al., 2004; Ferrari et al., 2007). However, the underlying mechanisms by which OGs induce and activate defenses are not clearly defined. To identify new components of the early response to OGs, I carried out full-genome expression profiling of Arabidopsis RNA isolated from OG-treated seedlings using Affymetrix ATH1 GeneChips.

The seedling system has significant advantages compared to using mature plants or protoplasts. It is sterile and does not contain potentially pathogenic organisms (insects, spores, etc..), which could activate defense mechanisms and confound the results. The plantlets are ready to use in 10 days and do not take as much space as 4 weeks old plants. Moreover, infection with bacterial pathogens can be carried out by simply adding an inoculum of bacteria to the medium.

Nevertheless the seedling assay also has some limitations. For example, it is not possible to infect seedlings growing in liquid with *B. cinerea*, because the fungus will grow using resources from the medium instead of infecting the plant, and the plant will die from nutrient deprivation but not from infection. However, we find using this seedling system, that a significant fraction of the genes activated by the OGs are also activated by *B. cinerea* in adult plants, which suggests that an OGs-mediated defense response is a key aspect of *B. cinerea* infection. Moreover genes identified using the seedlings assay system are also induced in adult plants. The seedling system is thus similar enough to the adult plant system to be a good alternative for studying defense responses.

The microarray experiment analyzing seedlings treated with 200µg/ml OGs for 1 and 6h includes one technical replicate for each treatment. The GeneChip is a very sensitive technology and it was imperative to determine that within the same experiment, reproducible data could be obtained. Although I checked the integrity of the RNA after it was isolated, many steps occur before the GeneChip data are obtained. Each stage of the process is a potential source of variation, including the numerous RNA manipulations and the involvement of complex machinery (Thermal cycler, oven, fluidic station and scanner).

During my tenure in the Ausubel lab, I processed a large number of RNA samples for GeneChip analysis including RNAs from plants infected with *B. cinerea, Erisyphe orontii* (renamed *Golovinomyces orontii*), *and P. syringae*, from plants treated with different MAMPs, and from plants subjected to herbivory by the caterpillar *Trichoplusia*

ni. For several of these experiments, variation due to sample processing was assessed by hybridization of technical replicates, prepared from the same RNA sample. The analyses of these technical replicates showed that I obtained a high level of reproducibility between chips.

Although many of these transcriptional profiling experiments were not part of my Ph.D. project, I was interested in MAMP signal transduction and I started to work on analyzing the data from the microarray experiment involving treatment of seedlings with OGs for 1 or 6 hours. This analysis revealed a transient OG-elicited response that included the activation of a set of genes encoding P450s involved in the biosynthesis of indolic compounds such as *CYP71B15*, *CYP71B13* (camalexin biosynthesis) and *CYP83B1* (indolic glucosinolate). Two of these CYP genes are also strongly activated by *B. cinerea* infection in adult plants. I also identified two cytochrome P450-encoding genes, *CYP81F2* and *CYP82C3*, with unknown roles. They are the most highly induced P450s after 1h OGs treatment.

Through the study of the response to OGs, I became interested in both the regulation of these two CYP genes and their function in defense. I accumulated evidence that the two genes have different roles in the response to OGs. First, *CYP81F2* is co-up-regulated in response to OGs and *B. cinerea* whereas *CYP82C3* does not show any significant change with the fungus. In addition, the profile of expression of the two genes is not identical over time. *CYP81F2* expression is sustained in comparison to *CYP82C3* expression in response to OGs.

CYP81F2 is part of a family that includes 3 others members in *Arabidopsis thaliana*, with 64%, 68% and 72% amino-acid identity to CYP81F2 in their sequence. CYP82C3 is part of a family that contains 2 other members that share 80% or more amino acid identity and, moreover, the corresponding genes are clustered together on chromosome 4. This suggests that the CYP82C family members could be functionally redundant or belong to the same pathway. In contrast, *CYP81F2* is alone on chromosome 5 and its family members are on chromosome 4, suggesting that CYP81F2 may have a function distinct from them.

It has been reported that OGs are involved in two categories of biological responses: plant defense, and plant growth and development (Darvill et al., 1992; Ridley et al., 2001). Interestingly *CYP81F2* is induced by *B. cinerea* and OGs, whereas the regulation of *CYP82C3* is not overlapping in either experiment. This observation leads to the hypothesis that CYP81F2 functions as part of OG-mediated plant defense, whereas CYP82C3 may function in OG-mediated plant growth and development. Further study on the role and regulation of these genes is the subject of the last chapter in this thesis.

Additionally I accumulated evidence that the OG defense response in seedlings is similar to the OG response in 4-week old plants. Global expression analysis showed OG-elicited gene expression had almost returned to the basal level a few hours after OGs treatment and that the structure of OGs is critical to be perceived and to trigger a response. Any change in OGs length, methylation, esterification or conformation, could affect recognition of OGs. Oligosaccharides are biochemically very reactive molecules. Many hypotheses could explain why the active life of OGs does not extend more than 1h

(Figure 1-3). It was reported that about 30% of OGs bind rapidly to suspension-cultured tobacco cells, suggesting that soluble OGs could be modified or fragmented into biologically inactive OGs, presumably by enzymes such as polygalacturonases present in the growth medium (Mathieu et al., 1998). However, as shown in Figure 1-4, I could not detect any polygalacturonase activity in the medium. Another hypothesis is that OGs elicit a transient response because plant cells become desensitized to any further elicitation by OGs. A phosphorylation event may be involved in this refractory state (Navazio et al., 2002).

Recently, Cabrera and colleagues reported that OGs in solution containing calcium/sodium could change conformational state from single-isolated chains into calcium-associated multimeric chains. OG dimers called eggboxes could bind to a WAK1 receptor (wall associated kinase) and induce an increase in extracellular alkalinization (Cabrera et al., 2008). One of the earliest responses of Arabidopsis to OGs is the extracellular alkalinization with an increase of Ca^{2+} (Darvill et al., 1992; Lecourieux et al., 2002; Navazio et al., 2002; Hu et al., 2004; Romani et al., 2004). The rapid increase of Ca^{2+} in the medium induced by OGs could increase OG multimerization in the medium resulting in a biologically inactive conformation of OGs. The increase in calcium ions in the medium occurs within seconds to 5 min, consistent with our observation that OG-eliciting activity is rapidly lost.

Even though the OG-elicited response is transient, it is sufficient to partially protect seedlings from *P. syringae* infection. This reduction of bacterial growth was also

transient, and we cannot distinguish if it is an effect due to the rapid diminution of active OGs or because at a certain titer the bacteria overwhelm the seedling defenses.

Finally, I show that the OG response, at least with respect to the two P450s tested, is localized in the shoots and that OGs induce rapid transcriptional changes which are independent of the salicylic acid, jasmonic acid, and ethylene signaling pathways.

Chapter 2.

Arabidopsis defense response to endogenous versus exogenous elicitors

ABSTRACT

OGs and Flg22 are two MAMPs that have been shown to activate common cellular responses in plants. Although the two elicitors are detected by different receptors, transcriptional profiling of seedlings shows that they initially activate a similar early response. These early responses are both fast and transient and involve activation of defense signaling pathways, such as JA-associated processes. The response to Flg22 is somewhat stronger in both the number of genes differentially expressed and the amplitude of change. Significantly, at later time points, the response to each elicitor is markedly different: in the case of OGs, expression of most genes returns to basal levels by 3 hours post treatment (hpt). In contrast, the response to Flg22 continues to be robust at 3 hpt. The magnitude of induction of individual genes is in both cases dose dependent, but even at very high concentrations, OGs do not induce a response that is identical to that seen with Flg22. Flg22, but not OGs, induces salicylic acid-associated defenses including expression of *PR1*, stimulates secretory pathways, elicits callose deposition in seedling cotyledons, and induces senescence in seedlings. Interestingly, both OGs and Flg22 treatment reduces bacterial growth in seedlings (Chapter 1; Paper 1) (Songnuan et al., 2007). In adult plants, both elicitors can activate callose accumulation in adult leaves, depending on experimental conditions, but other responses, such as elevated *PR1* expression, are elicited only by Flg22. At early times, OGs and Flg22 activate common early defense mechanisms, but at later times different signal transduction pathways are activated leading to OGs- and Flg22-specific responses.

INTRODUCTION

Pectin-derived oligogalacturonides (OGs) and a synthetic peptide from a conserved domain of bacterial flagellin (Flg22) function as general elicitors of defense responses in a variety of plant species. Studies carried out in a number of labs have indicated that there is a significant overlap in the early responses to both elicitors, including changes in protein phosphorylation, activation of MAP kinases, induction of Ca^{2+} fluxes, ROS accumulation, stomatal closure, and transcriptional up-regulation of a suite of genes previously implicated in defense. Additionally, experiments in which plants were pre-treated with either of these elicitors prior to pathogen challenge indicate that OGs and Flg22 can activate defenses that contribute to limiting pathogen growth and/or development (Aziz et al., 2004; Zipfel et al., 2004; Ferrari et al., 2007).

MAMPs comprise many different classes of molecules. OGs and Flg22 are very different in their structure and origin. OGs are oligosaccharides that can be derived from plant cell walls either through the activity of microbial polygalacturonases or during plant development, whereas Flg22 is a proteinaceous molecule that is exclusively associated with bacterial microbes. The emerging idea is that microbe-associated molecular patterns (MAMPs) trigger a common set of responses that constitute what is referred to as basal resistance. In order to determine the extent of similarity in the response to Flg22 and OGs, we compared the FLARE (flagellin rapidly elicited) genes from Navarro and colleagues (Navarro et al., 2004) with OGRE (OGs rapidly elicited) genes identified in our laboratory (chapter 1) that are activated in seedlings by OGs after 1 hour and 6 hours. However, different experimental conditions such as plant tissue and age, environmental

conditions, duration of the treatment, etc. made it difficult to accurately assess the degree of similarity between responses to Flg22 and OGs in these experiments. I therefore conducted a direct comparison of transcriptional re-programming after exposure of Arabidopsis seedlings to OGs or Flg22 to gain a more comprehensive view of the extent of similarity and divergence in the responses to these two elicitors.

RESULT

1- Activation of Defense Response Pathways by OGs and Flg22 Elicitors in Arabidopsis Seedlings

Chapter 2; Paper 1 (Denoux et al., 2008)

Annex 2; Chapter 2; Paper 2 (Denoux et al., 2007)

Activation of Defense Response Pathways by OGs and Flg22 Elicitors in Arabidopsis Seedlings

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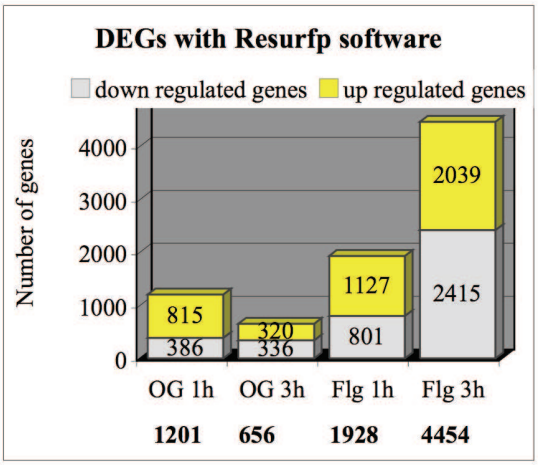
Following, are additional data about the activation of defense response pathways by OGs and Flg22 in Arabidopsis seedlings. They were not included or referred to as "not shown" in Chapter 2; Paper 1 (Denoux et al., 2008) or in (Denoux et al., 2007)

2- Comparison of the sets of Differentially Expressed Genes (DEGs) selected by the ReSurfP or Rosetta Resolver algorithms

To determine whether the sets of genes identified as differentially regulated by Flg22 and OGs were dependent on the statistical algorithm used to analyze the transcriptional profiling data, we compared the results obtained using two software programs, Rosetta Resolver (Waring et al., 2001) and ReSurfP (Gopalan, 2004). A major difference in the algorithms is the level of intensity data used for comparison of samples: the Resolver software calculates an average intensity for each probe set prior to comparing values from different samples, whereas ReSurfP compares intensity values for each probe of the different samples.

As in the Resolver analysis reported in Chapter 2; Paper 1 (Denoux et al., 2008), analysis with ReSurfP indicates that the response to OGs declines in few hours (1201 DEGs at 1h versus 656 at 3h), whereas the response to Flg22 is more sustained (1928 DEGs at 1h versus 4454 at 3h). Additionally, Flg22 induces a stronger response than OGs, at 1hour (1928 Flg22 DEGs versus 1201 OGs DEGs) and at 3hours (4454 Flg22 DEGs versus 656 OGs DEGs (Figure 2-1)). As in the case of the Resolver analysis,



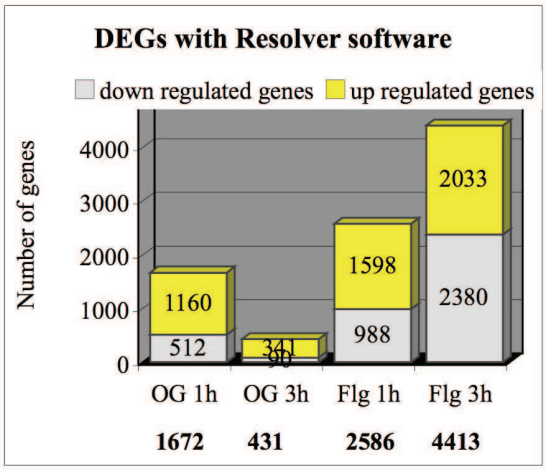


Figure 2-1: Comparison of the sets of Differentially Expressed Genes (DEGs) selected by ReSurfP or Rosetta Resolver algorithm.

Numbers at the bottom of the treatment are the total DEGs of the corresponding treatment.

ReSurfP analysis showed that most of the genes that were activated by OGs were also responsive to Flg22 (1025 of 1201 at 1 hour, 611 of 656 at 3h).

Although the overall trends were consistent between the two algorithms, there were noticeable differences in the number of genes that were considered to be significantly changed after treatment (Figure 2-1). Comparison of the genes picked by each algorithm indicated that ReSurfP selections met more stringent probability criteria. On the other hand, the majority of genes picked only by ReSurfP were not picked by Resolver because they failed to meet the 2-fold change threshold applied in the Resolver analysis (Table 2-1). The expression of the genes presented in Table 2-1 (with the exception of *CYP82C3*) has been evaluated by Real-time PCR. All were confirmed to be induced by Real-time PCR (Denoux et al. 2008, Figure 2).

Both algorithms indicated the same overall pattern of response to the two elicitors, suggesting that relaxing either fold-change or probability criteria does not alter the conclusion that OGs and Flg22 elicit initially similar responses that diverge over time.

3- Lack of induction of classic SA and JA elicited marker genes in seedlings by Flg22 or OGs

In Denoux et al. 2008, we examined the kinetic behavior of elicitor-induced genes by assaying expression of selected genes over a long time course, from 0.5 to 48 h. Gomez-Gomez and colleagues reported the specific induction of *PR5* and *PDF1.2* expression after Flg22 treatment of Arabidopsis (Gomez-Gomez et al., 1999) and Moscatiello and

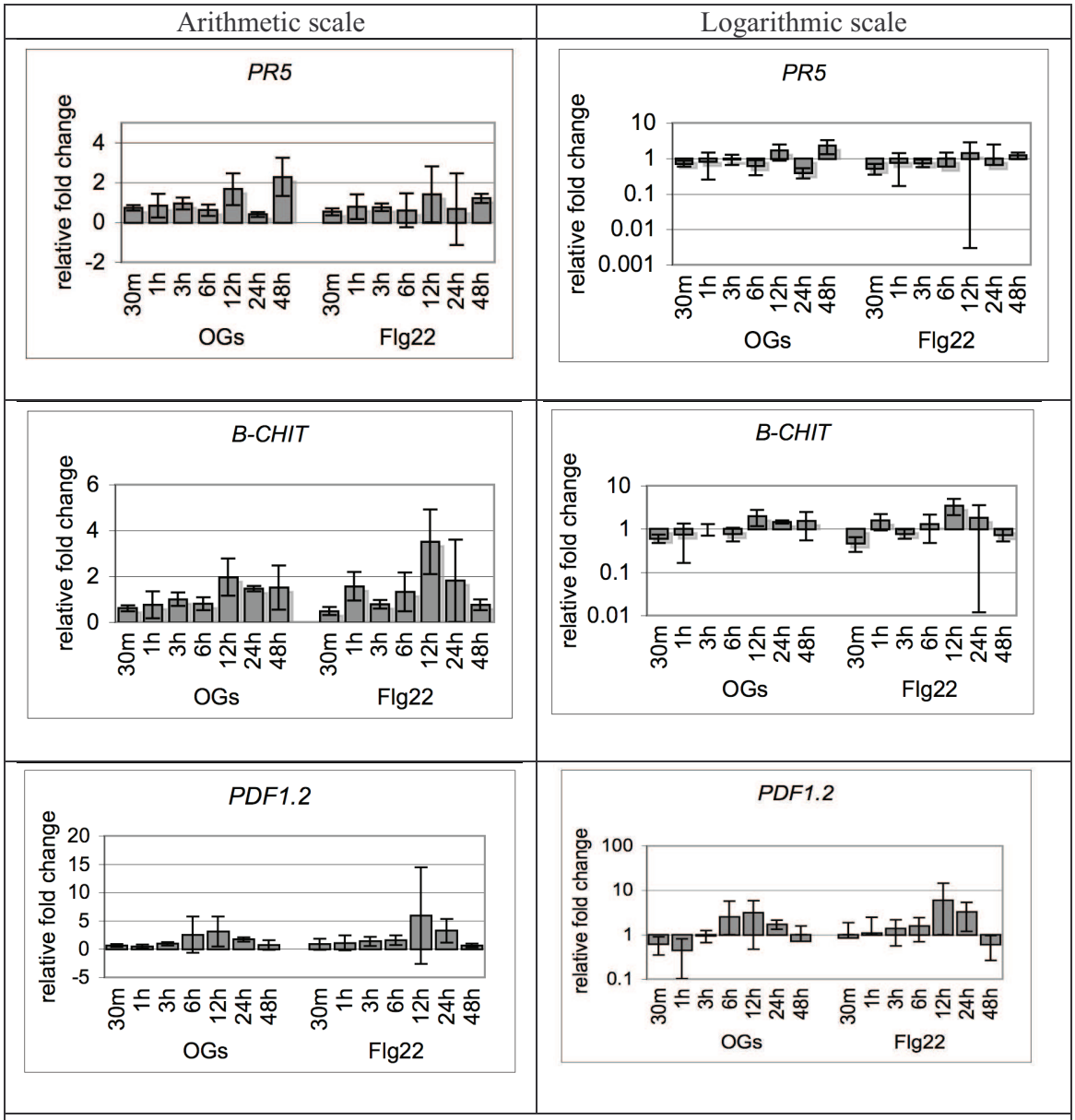


Figure 2-2: Pathogenesis-related genes *PR5*, *B-Chitinase*, and *PDF1.2* were not significantly induced by OG or Flg22 elicitors

Col-0 seedlings were elicited with OGs or Flg22 as for the expression profiling experiments, and harvested at 30 min or 1, 3, 6, 12, 24, or 48 h. The fold change relative to water-treated samples was assayed by reverse-transcription–real-time PCR. Values shown are average and standard deviation of two independent experiments.

colleagues reported an increase in genes encoding enzymes for JA biosynthesis following OGs treatment of Arabidopsis (Moscatiello et al., 2006). Therefore, I assayed the induction of defense related genes including *PR5*, *B-CHIT* and *PDF1.2* by RT-qPCR (Figure 2-2). These data were not included in Denoux et al. 2008 because there was no significant and reproducible increase in expression levels of the three genes tested in the case of any of the treatments. There was also a lot of variability from experiment to experiment and it was difficult to assess where the variability was coming from: reaction mix, primers, or biological/technical replicates, etc. A variety of possibilities were tested without success. The data are shown in Figure 2-2 plotted both arithmetically and logarithmically. These data suggest that *PDF1.2* may be marginally induced at 12h after either OGs or Flg22 treatments, since the same pattern was observed in several experiments, but the data are not statistically significant. Interestingly, Aslam et al reported *PR1* expression following infiltration of leaves with Flg22, with a peak of induction at 12 hpt, but did not observe any induction with OGs (Aslam et al., 2009), consistent with the conclusions published in Denoux et al. 2008. Additionally, Aslam et al. demonstrated that *PDF1.2* is significantly induced at 12 hpt by both elicitors suggesting that the JA signaling pathway is up-regulated by both OGs and Flg22. Under some growth conditions, the level of endogenous ET is insufficient for induced expression of *PDF1.2,* which requires both ET and MeJA (Penninckx et al., 1998), and this perhaps accounts for the variable expression of *PDF1.2* in some of our experiments.

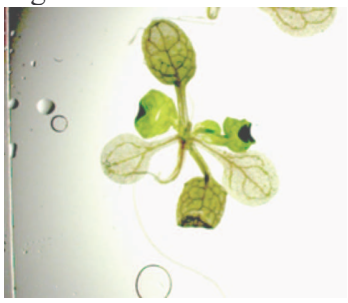
H_2O	OGs	Flg22
		

Figure 2-3. Flg22 but not OGs induces senescence and growth inhibition in seedlings

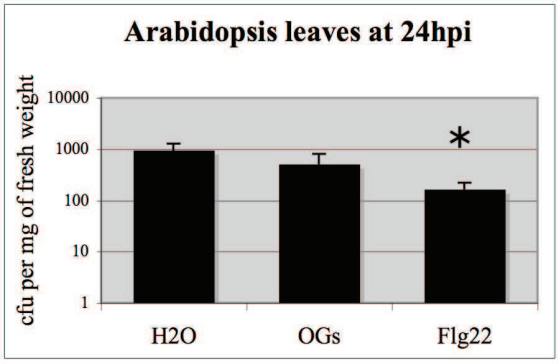
Seedlings grown in liquid culture for 10 days were treated with 50 µg/ml OGs or 1 µM Flg22 and photographed 4 days later. 4 wells with 5 to 10 seedlings per well were assayed per treatment. The samples shown are representative of seedlings from two independent experiments).

4- Flg22 but not OGs activates senescence and affects growth of Arabidopsis seedlings

Induction of a senescence program is a common response to pathogen infection (Gan and Amasino, 1997) and reducing nutrient availability to pathogens is a part of some defense mechanisms. Characteristic of senescing leaves is the reduction of photosynthesis-related genes (Morris et al., 2000) and breakdown of the chloroplast, where a large portion of the nitrogen in a leaf cell is stored (Gan and Amasino, 1997).

Our microarray data indicate clusters of genes that are down-regulated by Flg22 at 3 hours (but not by OGs) that are enriched for chloroplast protein synthesis, electron transport and plastid components, key enzymes of the photosynthesis system. Moreover, expression of secretory pathway proteins and transcription factors associated with senescence, including WRKY6 and WRKY53, (Robatzek and Somssich, 2001; Miao et al., 2004), are induced specifically 3 hours after Flg22 treatment. These data suggest Flg22, but not OGs, activates a senescence program. As shown in Figure 2-3, leaves exposed to Flg22 treatment exhibit chlorotic symptoms. These symptoms become visible several days after treatment, when Flg22 is no longer active in the medium and when new leaves are developing. Similar symptoms are observed in seedlings treated with exogenous SA (data not shown), except that the symptoms elicited by SA are more severe. The observation that Flg22 elicits a senescence-like response is consistent with published reports showing that senescing leaves are characterized by high levels of the SA-regulated *PR* gene *PR1* (Robatzek and Somssich, 2001). As shown in Denoux et al., 2008, Flg22 but not OGs activate SA-dependent pathways including amplified expression

A



B

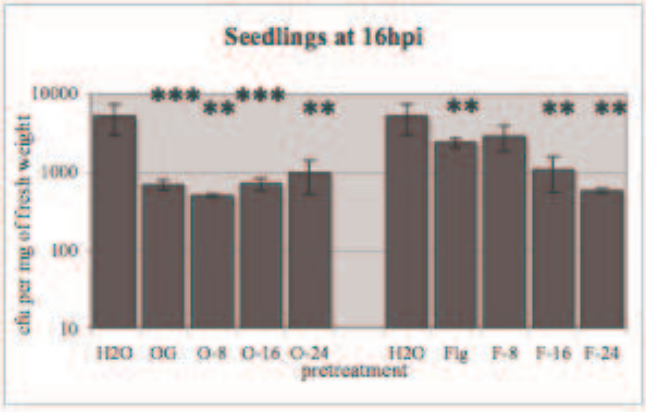


Figure 2-4: Elicitors differentially protect against *Pst* DC3000.

A. Bacterial count at 24 hpi, in leaves of 4 week old plants infiltrated with water (H_2O), 200□g/ml OGs or 1□M Flg22 and inoculated with *Pst* DC3000 OD_{600} = 0.0002 24h after treatment.
B. Bacterial count at 16 hpi, in 10 days old seedlings pretreated with water (H_2O), 50□g/ml OGs or 1□M Flg22 at different times (0, 8h, 16h, 24h) prior to inoculation with *Pst* DC3000 OD_{600} = 0.0002

(∗∗∗) Pvalue < 0.0001, (∗∗) Pvalue <0.001, (∗) Pvalue < 0.01, *t*-test.

of *PR1*. These results suggest that Flg22-elicited activation of SA-signaling triggers a senescence program. The activation by Flg22 of a senescence-like response triggered by SA-signaling is analogous to the activation of HR response like-PCD by avirulent pathogens. In addition to eliciting a senescence response, Flg22-treated seedlings exhibit a reduction in growth, which is correlated with the down-regulation of genes involved in growth and with the activation of senescence. Leaf, cotyledon, and root growth are all affected (Gomez-Gomez et al., 1999). The physiological consequence may be to conserve energy for the defense response. Treatment with OGs does not affect the growth of seedlings. From the transcript profiling analysis, Flg22 but not OGs regulate growth hormones such as gibberelin and abscissic acid (ABA). These two metabolic processes, senescence and growth inhibition, are probably co-ordinated and are both specific to the Flg22 response in seedlings.

5- Reduction of bacterial growth in seedlings pretreated with OGs or Flg22

As previously shown by Zipfel and colleagues, Flg22 protects plants from subsequent infection by *Pst* DC3000 in Arabidopsis leaves (Zipfel et al., 2004). Our collaborator Simone Ferrari and I tried to repeat the Zipfel experiment with OGs in adult plant and neither of us obtained any conclusive data (Figure 2-4A). In seedlings, however, I showed that OGs transiently reduced the bacterial growth. Interestingly, the level of protection with Flg22 increased with time of treatment whereas OGs-mediated protection peaked at 48 hours (Chapter 1; Paper 1)(Songnuan et al., 2007). The relatively small effect of OGs on limiting *Pst* DC3000 growth was not always easy to observe. It might

be due to the increase of H_2O_2 in the medium after recognition of the elicitor. Indeed, when the elicitor is added at the time of inoculation, the protective effect of OGs could be seen earlier than 24 hpi (Figure 1-5; chapter 1), a time point where we do not yet observe the Flg22 protective effect (Zipfel et al., 2004). Moreover, we could see at 16 hpi an optimum protective effect in seedlings pretreated 8h with OGs, whereas it was optimum in seedlings pretreated 24h with Flg22 (Figure 2-4B). Despite the difference in the kinetics of the MAMP-induced protection against subsequent bacterial infection, both OGs and Flg22 caused a significant reduction in bacterial growth in seedlings. The fact that adult plants were partially protected by Flg22 but not by OGs may be a consequence of the length of the MAMP pretreatment prior to bacterial inoculation.

6- Differential elicitation of callose deposition by OGs and Flg22

Callose is a sugar polymer of (1,3)-β-D-glucan subunits that is deposited at the site of attempted penetration by pathogens, acting as a physical barrier against colonization of the intercellular space (Maor and Shirasu, 2005). Formation of callose appositions is a target for suppression by bacterial virulence factors or secreted exopolysaccharides (DebRoy et al., 2004; Yun et al., 2006). Deposition of callose is induced by Flg22 in Arabidopsis seedlings (Gomez-Gomez et al., 1999). To determine whether OGs also induce this basal defense response, I analyzed callose deposition in seedlings and leaves of Arabidopsis.

Kinetics of callose deposition induced by infiltration of OGs and Flg22.

When adult leaves are infiltrated with OGs or Flg22 using a needle-less syringe, callose deposition is readily apparent starting about 12 hours after infiltration (Figure 2-5). No callose was induced in the water-treated (control) leaves. Interestingly, callose deposition elicited was different for the two MAMPs. Flg22 triggered deposition of small, thin dots uniformly spread in the infiltrated area. In contrast, OGs triggered the uneven deposition of callose spots of various sizes. These differences could be a consequence of how and/or where OGs and Flg22 are perceived by the plant. In any case, both Flg22 and OGs induced callose depositions, which could be visualized by 12 hours post treatment and was very strong at 18h.

Differential callose accumulation in seedlings and leaves.

Seedlings and leaves were treated or infiltrated, respectively, with OGs or Flg22 for 18h and stained for callose deposition (Figure 2-6A). Leaves infiltrated either with OGs or Flg22 exhibited multiple callose-containing depositions in wild type, but not in *pmr4* (callose synthase) leaves. As expected, in the *fls2* (flagellin-sensitive2) mutant, callose was detected after OGs infiltration, but not with Flg22. In contrast to leaves and in contrast to Flg22, no callose deposition was elicited by OGs in Arabidopsis seedlings (Figure 2-6B). As expected, callose was detected after Flg22 treatment in wt seedlings but not in *pmr4* or *fls2* mutants. In contrast to leaves where a small amount of staining was observed in water-infiltrated *pmr4* leaves, no staining was observed in *pmr4* seedlings. Taken together, these data indicate that OGs are able to activate callose

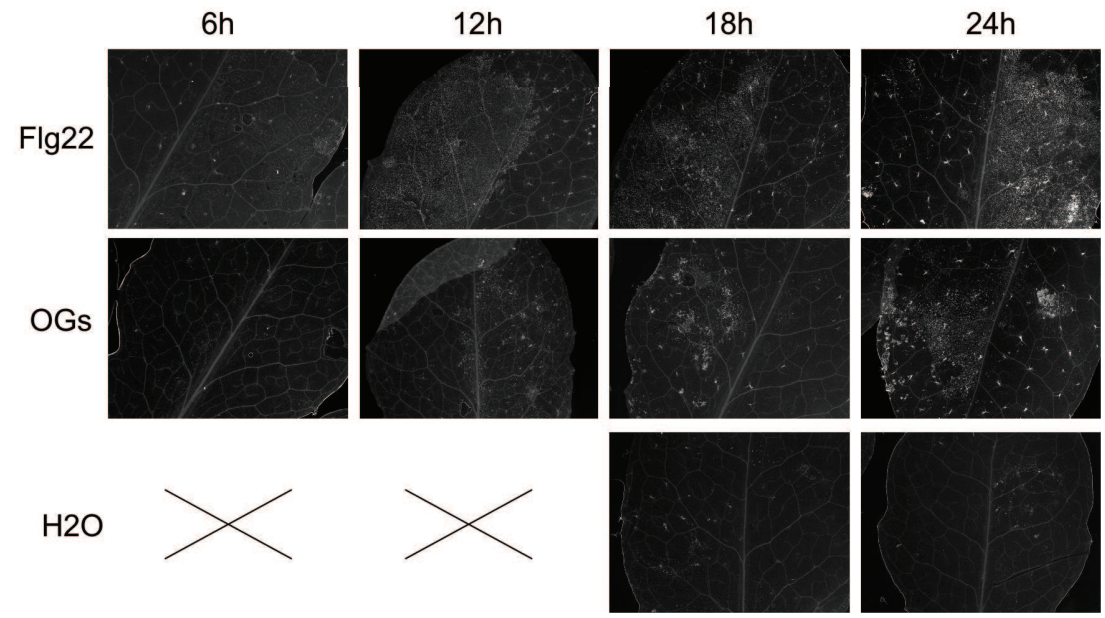


Figure 2-5: Kinetics of callose deposition induced by elicitor infiltration in Arabidopsis adult leaves

Flg22 and OGs solutions of 1µM and 200 ug/ml, respectively, were infiltrated into leaves of 4-week old Arabidopsis plants using a needleless syringe. Leaves were harvested at times indicated and stained with aniline blue for detection of callose. For each treatment, 4 leaves from individual plants were assayed. The experiment was repeated twice.

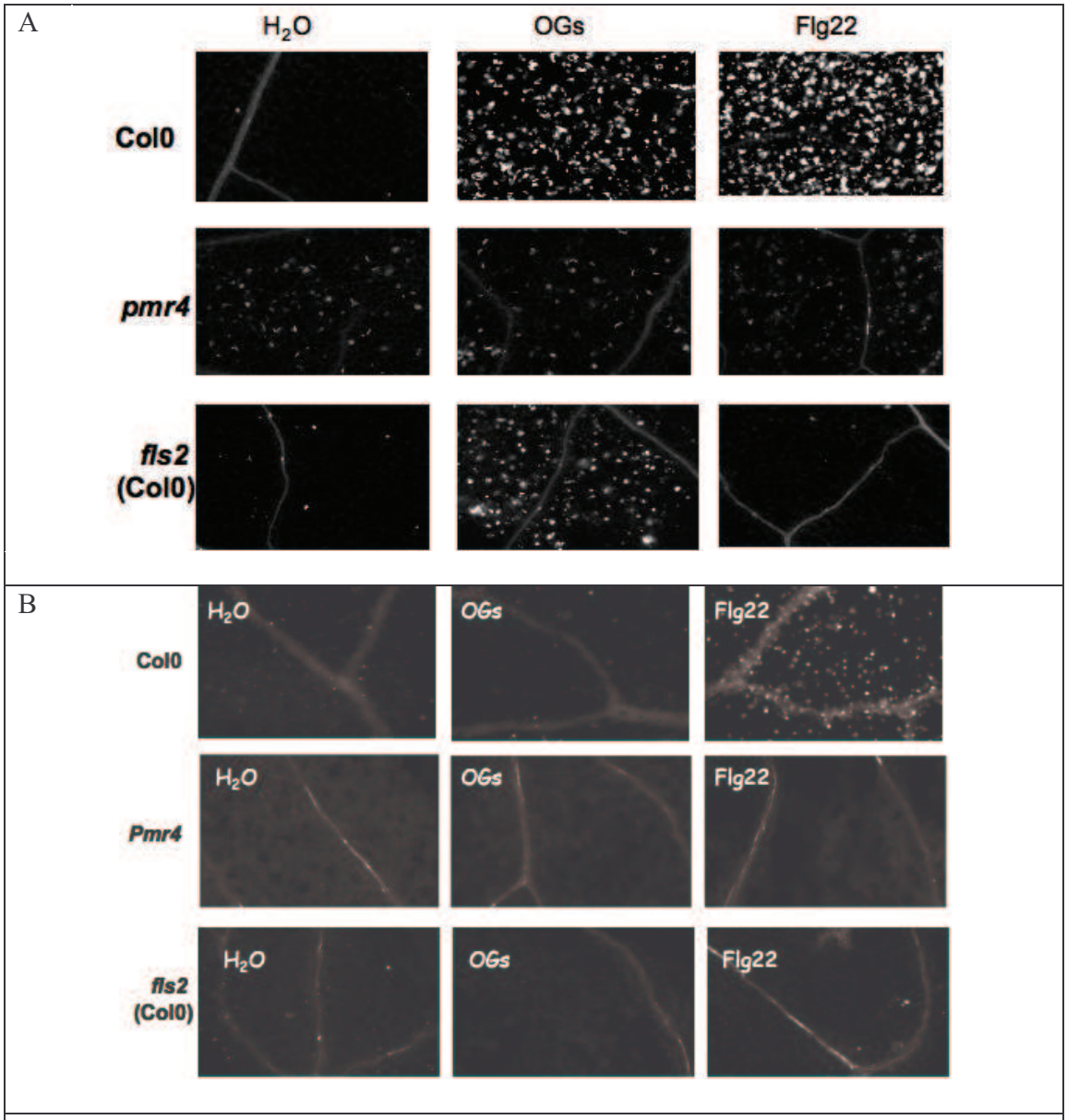


Figure 2-6: Differential elicitation of callose deposition by OGs and Flg22

A. Leaves of 4 week old plants were infiltrated with water (control), 200□g/ml OGs or 1□M Flg22 using a needle-less syringe.
B. 10 day old liquid-grown seedlings were treated with water (control), 200□g/ml OGs or 1□M Flg22 by addition of elicitor to the growth medium.
After 18 hours treatment, tissues were stained with aniline blue for callose detection by fluorescence microscopy.

deposition. The lack of OGs-induced callose deposition in seedlings suggests that OGs may not be transmitting the same signal in seedlings and adult plants. The difference probably does not come from the tissue type and age, more likely from the technique of elicitation and perception of OGs. In leaves, the infiltration method localizes the OGs directly in the intracellular space, whereas with the seedlings the OGs are added into the medium. In seedling medium, OGs chains could aggregate as multimers, which might decrease the level of active OGs able penetrate into the plant. This hypothesis suggests that a threshold of active OGs is necessary to activate callose deposition.

DISCUSSION

As described in many publications, a variety of general elicitors of the plant defense response trigger similar types of cellular changes, ion fluxes, production of ROS etc. I have focused my work on oligogalacturonides and a 22 amino acid flagellin peptide, two very different elicitors in both structure and origin. I carried out a direct transcriptional comparison of Arabidopsis seedlings treated by both elicitors. Furthermore, I extended the analysis to identify similarities and differences in the responses elicited by Flg22 and OGs. Taken together, these data give evidence that both elicitors activate a common basal early response. Later the responses diverge, but continue to share common features.

Although there is considerable overlap in the early signaling events elicited by Flg22 and OGs, differences occur in the modulation of kinetics and intensity of these events. For example the change in cytosolic and nuclear Ca^{2+} concentration induced by both OGs

and Flg22 varied in magnitude, timing, and pattern (Lecourieux et al., 2005). Moreover, although both elicitors activate the same MAP kinases, including MPK6 (Nuhse et al., 2000) and MPK3 (Denoux et al., 2008), the kinetics of activation is different. In transcripitonal profiling analyses of OGs and Flg22-treated seedlings, we found that both elictors induce highly correlated responses at 1h, but the magnitude of the response is lower for OGs. At later times, the responses show considerable differences, but continue to share some common features. As another example, the *ERF1*, *PGIP1* and *CML41* genes are induced following treatment with either elicitor but with different kinetics and at different intensities. Overall, the induction of common early events by OGs is faster, but at a lower level and more transient than the Flg22 response.

As soon as 3 hpt, the differences in levels of gene expression elicited by the two elicitors is apparent. Most of the genes differentially expressed by OGs return to basal levels within 3 hours, whereas the response to Flg22 continues to be robust for many hours. The response to Flg22 is somewhat stronger in both the number of genes differentially expressed and the amplitude of change. Among the differences, Flg22 stimulates secretory pathways, senescence and induces salicylic acid associated defenses, including amplification of *PR1* expression. Additionally, Flg22 induces callose deposition in both seedlings and in adult leaves. Interestingly, with OGs, callose could be detected only after infiltration of leaves but not following treatment of seedlings. I also did not detect any *PR1* expression after OGs treatment, whether OGs were added to the seedling medium or infiltrated into adult leaves. The different biochemical properties of OGs and Flg22 may be responsible for their different signaling properties in the seedling system. The MS medium contains a lot of ions, specially calcium and sodium. Following

gray: P > 0.01	Fold Change	Fold Change	Fold Change	Fold Change
Primary Sequence Name	**OGs 1h**	**OGs 3h**	**Flg22 1h**	**Flg22 3h**
transcription factor; regulator of indole glucosinolate biosynthesis				
MYB51	43.573	5.253	47.141	23.831
camalexin biosynthesis				
CYP79B2	4.564	5.506	6.468	29.529
CYP79B3	-1.244	2.640	-1.121	3.496
CYP71B15/PAD3	3.880	11.467	5.831	16.913
CYP71A13	1.480	2.115	1.740	13.761
indole glucosinolate biosynthesis				
CYP79B2	4.564	5.506	6.468	29.529
CYP79B3	-1.244	2.640	-1.121	3.496
CYP83B1	4.568	5.523	4.604	10.184
SUR1	1.449	2.920	-1.042	4.209
UGT74B1	1.717	2.508	1.258	4.449
atST5a	3.097	3.765	2.541	6.458

Table 2-2: Expression of genes involved in biosynthesis or regulation of indolic defense compounds after treatment with OGs or Flg22 elicitors.

Fold-change values are highlighted in gray if the probability for differential expression did not meet the threshold of P-value <= 0.01.

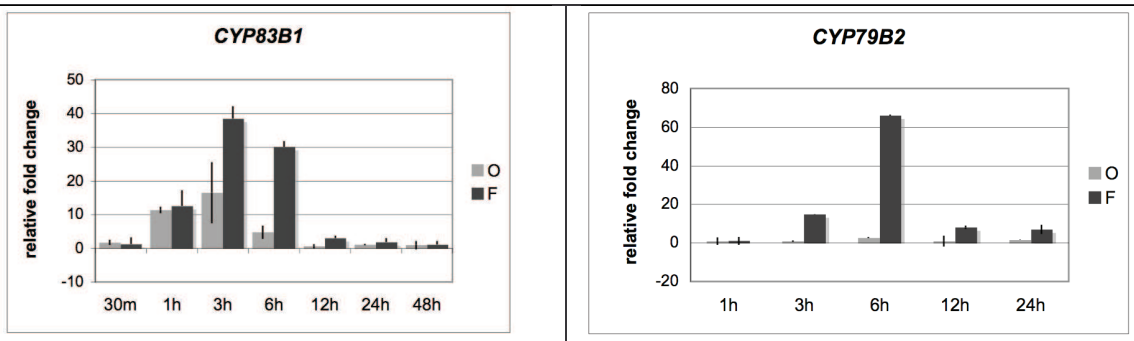


Figure 2-7: Induction of genes involved in indole glucosinolate biosynthesis.

Col-0 seedlings were elicited with OGs or Flg22 as for the expression profiling experiments, and harvested at 30 min or 1, 3, 6, 12, 24, or 48 h.
The fold-change relative to water-treated samples was determined by reverse-transcription–real-time PCR. Values shown are average and standard deviation of two independent experiments.

OG perception, the extracellular calcium concentration can increase even more due to Ca^{2+} efflux. The increase in Ca^{2+} concentration could cause OGs to aggregate and form calcium-associated multimer chains, thereby becoming totally inactive for signaling (Cabrera et al., 2008).

In spite of the possibility that OGs may aggregate in the seedling system, it appears evident that OGs are recognized and transmit a signal. As small as this signal may be compared to Flg22, both elicitors induce expression of some later genes in seedlings, including genes that encode enzymes involved in the biosynthesis of indolic compounds (Table 2-2 and Figure 2-7). Moreover, Aslam and colleagues found that both Flg22 and OGs induce a jasmonic acid pathway leading to *PDF1.2* expression (Aslam et al., 2009).

Most of the genes differentially activated by OGs are also activated by Flg22. Furthermore, all defenses or protection effects elicited by OGs are also elicited by Flg22. In adult leaves infiltrated with elicitor, we could detect a callose response following either treatment. *Botrytis cinerea* lesion size was reduced after OGs or Flg22 and this resistance is mediated independently of salicylic acid, jasmonate or ethylene signaling but requires *PAD3* (Ferrari et al., 2007). Another common protective effect of the elicitors was against bacteria. In seedlings, both were able to limit *Pst DC3000* growth. All together, these data indicate that the defense responses elicted by OGs in Arabidopsis are a subset of the responses elicited by Flg22.

Although work by Aslam and colleagues found that OGs elicit a distinct response from Flg22, this was not reflected in our transcriptional profiling data. For example, Aslam et al. report that OGs but not Flg22 elicit expression of *PAL1* (phenylalanine ammonia lyase 1), a key enzyme of the phenylpropanoid pathway, which might

contribute to an early induced, cell wall-based defense mechanism and occurs in response to non-adapted and non-host bacteria (Mishina and Zeier, 2007). This gene in our array was induced by OGs, 3.6 fold at 1h and 3.5 fold at 3h (at a P-value ≤ 0.0001) as well as by Flg22, 4.4 fold at 1h and at 20.76 fold at 3h (at a P-value ≤ 0.0001). I did not evaluate *PAL1* induction by quantitative PCR because independent reports also indicate that *PAL1* is induced by both Flg22 (Asai et al., 2002; Navarro et al., 2004) and by OGs (Aziz et al., 2004; Hu et al., 2004). To determine that in our data the probe detected really corresponded to the *PAL1* gene, I blasted all the probes set sequences from the Affymetrix GeneChip probe set ID (263845_at) avalaible on their website, corresponding to the *PAL1* gene name, and found that they were all *PAL1*-specific. The difference with Aslam and colleagues is that they infiltrated leaves from short-day grown plants.

In agreement with our data, Aslam et al. 2009 found that Flg22, but not OGs, induced *PR1*. It is not always easy to reach any straight-forward conclusion by comparing transcriptional profiling data, especially if one takes into account the different experimental condition (plant tissue and age, environmental condition, duration of the treatment, treatment in itself, procedure, etc.). In another example of apparent conflicting results, because of different experimental condition, Hu and colleagues found that OGs induce expression of *PR1* in seedlings (Hu et al., 2004). However, under a variety of different experimental protocols and conditions, I could not replicate this result in seedlings. In all cases, I found that Flg22 but not OGs induced *PR1* expression. One possible reason for the discrepant results may be that we use OGs isolated in Giulia De Lorenzo's laboratory at the University of Rome with a DP of 10 to 15, whereas Hu and

colleagues used a different purification protocol. Their experiments were also carried out on 3-week old ecotype Landbserg (Ler) seedlings on agar plates.

CONCLUSIONS

Although OGs and Flg22 are not detected by the same receptor, both are recognized by the plant and perceived as a component of a potential pathogen invasion, leading to rapid activation of an immune response. This rapidly elicited immune response is a common early defense that targets both host and non-host pathogens. Within this early response, the magnitude and kinetics of the signal influence the expression of different downstream defense networks. As soon as 3h after elicitation, we observe that the transcriptional responses to OGs and Flg22 are considerably divergent, leading to MAMP-specific defense responses. As a general rule, it appears that the nature of the second adaptive and more specific response is MAMP-specific.

The Flg22 downstream responses include: secretory pathways, growth inhibition, activation of senescence process, salicylic acid associated defenses including *PR1* expression, callose deposition, JA associated defense, and activated biosynthesis of indolic coumpounds (camalexin and indole-glucosinolates). The OGs-elicited secondary or late response appears to be a subset of the Flg22 response, and includes callose deposition (in leaves), JA associated defenses, and activated biosynthesis of indolic compounds (camalexin and indole-glucosinolates).

Chapter 3

Characterisation of *CYP81F2*, gene marker of early Arabidopsis immune response

SUMMARY

In the course of carrying out transcriptional profiling analysis of the response of Arabidopsis seedlings to oligogalacturonides (OGs), I detected in the pool of the most highly induced genes induced at 1h, two cytochomes P450, *CYP81F2* and *CYP82C3*. Several other CYP450 genes were also significantly upregulated, including *CYP71B15* (*PAD3*) that is involved in camalexin biosynthesis (Zhou et al., 1999; Schuhegger et al., 2006). Several other genes known to be involved in plant defense were also up regulated by OGs at 1 hour as shown in Table 1-2.

CYP81F2 and *CYP82C3* were chosen as representative defense-related genes to further study the Arabidopsis response to OGs in seedlings. Their functional role was unknown until recently. Through the study of the response to OGs, I became interested in both the regulation of these two CYP genes and their function in defense. In the end, I decided to focus on *CYP81F2* because more genetic tools were available, such as insertion line mutants corresponding to this gene that were not available for *CYP82C3*. My work on the role and regulation of *CYP81F2* is summarized here and in Clay et al., 2009 (which is appended to the thesis as an annex) but the data not shown in detail.

The gene encoding the cytochrome P450 CYP81F2 is induced rapidly in Arabidopsis seedlings in response to a variety of MAMPs including lipopolysaccharide (LPS), flagellin (Flg22), chitin, and EF-Tu (Elf18). *CYP81F2* expression is not localized to a particular tissue following MAMPs elicitation. *CYP81F2* is also activated in response to a variety of pathogens. It is induced early in response to bacterial (*P.s syringae* pv. *tomato* DC3000 and *P.syringae.* pv. *phaseolicola* 3121; 18hpi) and fungal (*Golovinomyces orontii;* 6 and 12 hpi) infection. Interestingly, *CYP81F2* expression returns to basal levels in 24 hours following induction by *P. syringae* DC3000, a virulent pathogen, but not by *P.syringae* 3121, a non-host pathogen. This suggests that *CYP81F2* could be a target of DC3000 type III effectors. This hypothesis needs to be confirmed with the use of the DC3000 *hrcC* mutant that is defective in type III secretion.

In the case of *G. orontii*, the expression of *CYP81F2* also returns to a basal level 24h after *G. orontii* inoculation; however, later (3, 4 and 5 dpi) it is expressed strongly again. It is known that *G. orontii* also produces effectors that can suppress the host defense system (Micali et al. 2008). *CYP81F2* expression was also localized around lesions elicited by *B. cinerea,* and at the site of egg deposition by the leaf minor fly *Scaptomyza graminum*. All these data suggest the involvement of CYP81F2 in Arabidopsis basal defense.

Despite its central role in defense, however, *CYP81F2* expression in response to Flg22 is independent of the SA-, JA- and Et-signaling pathways, but partially dependent on the MYB51 transcription factor that regulates several cytochrome P450 genes

involved in glucosinolate biosynthesis.

Seedlings deficient in the expression of *CYP81F2* (two independent insertion lines) have interesting phenotypes. They are defective in callose deposition in response to Flg22 and have a low level of 4-methoxy-indole-3-methyl-glucosinolate (4MI3M) compared to wild-type plants (Annex 3), (Chapter 3; paper1) (Clay et al. 2009). The lower accumulation of 4MI3M in these lines is accompanied by a high level of indole-3-methyl-glucosinolate (I3M). This aberrant glucosinolate profile is also seen in adult plants (Bednarek et al., 2009) and in roots (Bednarek unpublished data). These results suggest that CYP81F2 is required for the 4 methoxylation of indolic glucosinolates, which is required for callose formation in response to Flg22 (Annex 3) (Chapter 3; paper1) (Clay et al. 2009).

The *cyp81F2* mutant line does not show a significant phenotype with respect to herbivory by *Trichoplusia ni* (cabbage looper) caterpillars or susceptibility to *P. syringae* infection. Interestingly, however, the *cyp81F2* mutant is more resistant to the fungal pathogen *Fusarium oxysporum* and more sensitive to both *G. orontii* and *B. cinerea.* The aberrant production of glucosinolates in *cyp81F2,* such as the over production of I3M, is presumably responsible for the *F. oxysporum* resistance. In contrast, the defense response activated by the CYP81F2 pathway is required for resistance to both *G. orontii* and *B. cinerea..*

Further work on the biochemical characterization of CYP81F2 is being carried ou in Danièle Werck-Reichhart's laboratory.

General Conclusion

DISCUSSION / CONCLUSION

Perception of potential pathogen invasion plays an important role in the activation of the plant immune response. Arabidopsis can recognize molecules that characterize microbes or plant-derived molecules generated at early stages of infection such as oligogalacturonides (OGs). I was particularly interested in self-derived molecules like OGs that signal danger and trigger a variety of defense responses that enhance resistance against *B. cinerea* and other pathogens. In this study, I identified new components of OGs signaling and response pathways.

For my studies of the OG response, I used a seedling liquid assay, which has significant advantages compared to using mature plants grown in soil or protoplasts. The seedling growth conditions are sterile, the seedlings do not take much space, and the plantlets are ready to use 10 days after germination. Moreover, bacterial infection or treatment with elicitors can be carried out by simply adding an inoculum of the bacterium or elicitor to the medium. This seedling system assay has some limitations. Infection of seedlings growing in liquid is mostly restricted to bacteria. Fungi such as *B. cinerea* will grow using resources from the medium instead of infecting the plant. Another limitation I discovered using this system was a significant amount of variability in the levels of activation of reporter genes from one experiment to the next as monitored quantitatively

using real-time PCR.

Despite this variability, in collaboration with Simone Ferrari (Universtiy of Rome), I found that there is a very significant overlap between genes activated in the seedlings system by OGs and in whole plants by *B. cinerea* as determined by global transcriptional profiling anlaysis. These data indicated that despite some limitations, the seedling system is a valid model in which to study the plant defense response. Moreover, these data showed that activation of OG response pathways is a major way that adult plants respond to *B. cinerea* infection. This latter conclusion is consistent with the observation that *B. cinerea*-encoded polygalacturonases, which hydrolyze pectin into OGs, are *B. cinerea* virulence factors.

Additional transcription profiling analysis that I carried out showed that OGs initially activate an early response (within 1 hour) that is very similar to the response elicited by Flg22. Flg22 is a synthetic 22 amino acid polypeptide that corresponds to bacterial flagellin and the two elicitors are detected by different types of receptors.

Among the genes activated early after OGs and Flg22 treatment, two cytochrome P450s, *CYP81F2* and *CYP82C3,* are highly induced. Monitoring the expression of these genes and other markers genes such as *WRKY40* in a variety of defense-related mutants suggested that the rapid induction mediated by OGs and Flg22 is independent of SA, JA, or Et signaling pathways. At later times, the response to the two elicitors is characterized by activation at early stages of multiple defense signaling pathways, particularly JA-associated processes and in the case of Flg22, SA.

Although Flg22 and OGs activate many of the same genes, especially early after induction, there are differences between OGs and Flg22 in the kinetics and intensities of gene expression. Thus, transcripitonal profiling analysis of OGs and Flg22-treated seedlings showed highly correlated responses at 1h, but the magnitude of the response was lower for OGs. At later times, the responses show considerable differences, but continue to share some common features. For example, the *ERF1*, *PGIP1* and *CML41* genes are induced following treatment with either elicitors but with different kinetics and at different intensities. Other genes that were induced by both elicitors were the JA responsive gene *PDF1.2* and genes that encode enzymes involved in the biosynthesis of indole glucosinolates and camalexin.

In addition to eliciting the expression of many common genes, both OGs and Flg22 treatment elicited protection against pathogens. Lesion size following infection with *Botrytis cinerea* was reduced in adult plants pre-treated with OGs or Flg22 and elicitor pre-treatment limited growth of *Pst DC3000* in seedlings.

A major difference between OGs and Flg22 was that the changes elicited by OGs were much more transient than the Flg22 response. Thus, most of the genes differentially expressed by OGs return to basal levels within 3 hours, whereas the response to Flg22 continues to be robust for many hours. Other differences were that Flg22 stimulates secretory pathways, senescence, and induces salicylic acid associated defenses, including amplification of *PR1* expression. Additionally, Flg22 induces callose deposition in both seedlings and in adult leaves, whereas OGs only elicit callose deposition after infiltration

of leaves but not following treatment of seedlings. The explanation may be that OGs aggregate in the seedling system, reducing their effective concentration. However, I did not detect any OG-elicited *PR1* expression, whether OGs were added to the seedling medium or infiltrated into adult leaves.

In summary, my work to identify similarities and differences in the responses elicited by Flg22 and OGs showed that both elicitors activate a common basal early response. Later the responses diverge, but continue to share common features. The different ways that Flg22 and OGs are perceived and their very different biochemical properties may be responsible for some of these differences, such as the kinetics of gene activation and the magnitude of the response. Overall, the Flg22 responses appear more extensive than the OGs response and that the defense responses elicted by OGs in Arabidopsis are probably a subset of those elicited by Flg22.

Finally, in my study of the Arabidopsis response to OGs and Flg22, I extensively used two cytochome P450 genes, *CYP81F2* and *CYP82C3,* as markers of the response. These two P450s were the most highly induced genes after OGs and Flg22 treatment. Their expression is also activated by a variety of other MAMPs. Their expression in Arabidopsis seedling is independent of the SA-, JA- and Et-signaling pathways. CYP81F2 catalyzes a step required for the production of 4-methoxylated indolic glucosinolates that is necessary for callose formation in response to Flg22. The newly identified *CYP81F2*-dependent signaling pathway activated by MAMPs that leads to callose deposition is required for full resistance to *G. orontii* and *B. cinerea*.

Much prior research on plant defense mechanisms has focused on resistance mediated by effector/R protein interactions, which can prevent the spread of a pathogen from the initial site of infection. More recently, an appreciation of the role of MAMP-triggered defenses in limiting pathogen growth has developed as studies have shown that MAMP-pretreatment reduces subsequent infection and that MAMP recognition receptors contribute to effective defense responses. This work has addressed several aspects of innate immunity: one, to what extent MAMPs recognition might contribute to cellular reprogramming following pathogen challenge; two, how similar are responses to different MAMPs; three, how MAMP signaling interacts with the previously-defined defense-associated signaling mediated by SA, JA, and ET; and four, the function of a highly-induced, MAMP-responsive gene. Increasingly, studies from other labs are finding, as reported in this work, that specific elicitors induce overlapping but distinct responses, including activation of SA, JA, or ET signaling, thus potentially enabling plants to mount an early response that is tailored to the specific pathogen encountered.

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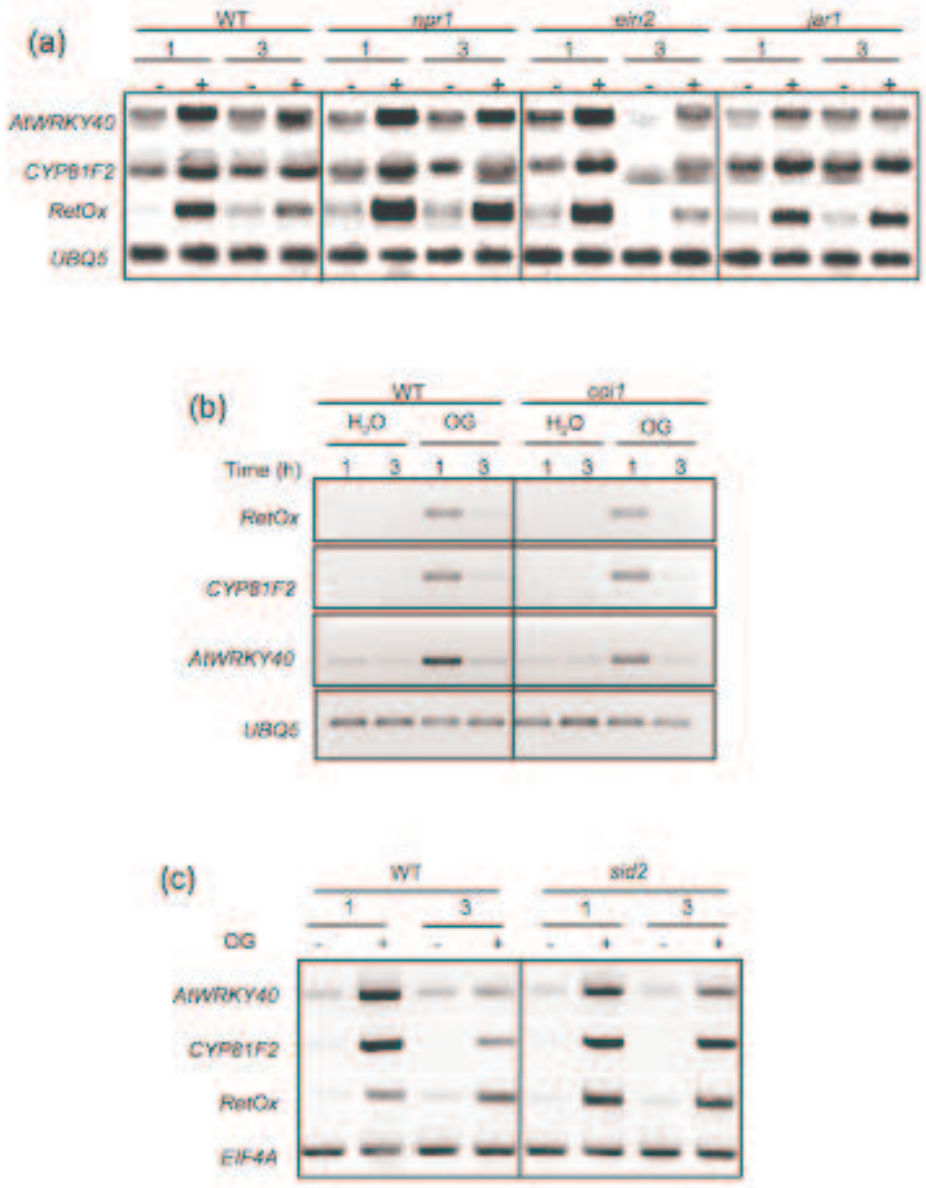
Annex

ANNEX 1



Supplementary Figure S3. Expression of selected marker genes in mutants impaired in SA, JA and ET signalling.
(a) Arabidopsis WT, *npr1*, *ein2* and *jar1* seedlings were treated with water (-) or OGs (+) for the indicated time (hours).
(b) Arabidopsis WT and *coi1* homozygous seedlings were treated with water (H_2O) or OGs for the indicated time (hours).
(c) Arabidopsis WT and *sid2* seedlings were treated with water (-) or OGs (+) for the indicated time (hours).
Expression of the indicated genes was analyzed by semi-quantitative RT-PCR, using the *UBQ5* gene as internal standard.

ANNEX 2

ANNEX 2 (chapter 2; paper 2)

Characterization of Arabidopsis MAMP Response Pathways

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Oligogalacturonides (OGs) and the bacterial flagellin peptide Flg22 are conserved general elicitors of the basal defense response in plants that are referred to as microbe- or pathogen-associated molecular patterns (MAMPs or PAMPs). Oligogalacturonides are produced by pathogen-derived polygalacturonase degradation of plant cell wall pectin and Flg22 is a synthetic 22-amino acid peptide derived from a conserved domain of bacterial flagellin. MAMPs trigger common cellular responses including ion fluxes, production of reactive oxygen species, MAP kinase activation, and induction of defense related gene expression. Pre-treatment of mature plants with MAMPs prior to pathogen challenge confers partial resistance to pathogen attack (Ferrari et al. 2007; Zipfel et al. 2004).

Transcriptional Profiling Analysis of MAMP-Elicited Seedlings

We have developed an Arabidopsis seedling assay system in which seeds are germinated directly in microtiter plates in liquid Murashige and Skoog basal medium, grown for 10 days, and then either treated with Flg22 or OGs or infected with an appropriate pathogen. A variety of experiments suggest that the seedling assay is a valid system to study pathogen infection and MAMP responses, including the observations that Flg22 elicits the production of callose in seedling cotyledons, that *Pseudomonas syringae* pv. *tomato* strain DC3000 kills seedlings, and that Flg22 and OGs treated seedlings are more resistant to DC3000 than untreated seedlings. These data indicate that the plant defense response elicited by MAMPS in 10-day old seedlings parallels the response in mature plants. The seedling assay and its validation are described in detail in a separate chapter in this volume (Songnuan et al. "A seedling assay for MAMP signaling and infection studies").

After validating the seedling assay, we carried out extensive transcriptional profiling studies utilizing Flg22 and OGs at early and intermediate time points. The rationale behind this experiment was to ascertain the extent of similarity in the transcriptional changes that are induced following treatment with two elicitors that differ in source (endogenous versus exogenous) and in structural classification (carbohydrate vs proteinaceous), and which have been demonstrated to differentially stimulate early cellular changes. The expression of several genes that were identified from expression profiling was subsequently assayed over an extended time-course to investigate the long-term patterns of transcriptional change resulting from exposure to elicitor. Both OGs and Flg22 triggered a fast and transient response that was initially quite similar. However, the early response to Flg22 was stronger in both the number of genes differentially expressed and the amplitude of change. The magnitude of induction of individual genes was in both cases dose dependent, but even at very high concentrations, OGs did not induce a response that was identical to that seen with Flg22. Our results indicate a highly

correlated early response but different overall outcomes. At 1 hour, transcriptional changes imply activation of SA, JA, and Et signaling circuits by both elicitors. In general, the initial MAMP-triggered immune response appears to lead to the rapid activation of early steps for an array of defenses, followed by either the cessation of the alert response or amplification of particular signaling pathways, depending on how the response was elicited.

These transcriptional profiling data significantly amplify a variety of published results. Full genome studies have been conducted previously in Arabidopsis treated with Flg22 (Navarro et al. 2004; Zipfel et al. 2004); chitin, a component of fungal cell walls (Ramonell et al. 2005); bacterial elongation factor Tu (EF-Tu) (Zipfel et al. 2006); oligogalacturonides derived from plant cell walls (Moscatiello et al. 2006); necrosis- and ethylene-inducing peptide 1 (Nep1) from *Fusarium oxysporum* (Bae et al. 2006); and necrosis- and ethylene-inducing peptide1–like protein 1 (NLP1) derived from *Phytophthora parasitica* (Qutob et al. 2006). Transcriptional profiling has also been conducted following infection of plants with *hrp*- mutants of *P. syringae,* which lacking a functional type III secretion system, are thought to elicit plant defenses solely via MAMPs (de Torres et al. 2003; Truman et al. 2006). Commonly, MAMP- induced early genes (within one hour) are functionally enriched for ones encoding antimicrobial compounds and for proteins involved in signal perception and transduction, including receptor-like kinases, transcription regulatory factors, kinases, and phosphatases (Moscatiello et al. 2006; Navarro et al. 2004; Zipfel et al. 2006; Zipfel et al. 2004). As well, a significant number of genes, which were previously shown to be required for basal or effector-triggered immunity, are transcriptionally regulated after exposure of plant tissue to elicitors (Navarro et al. 2004; Ramonell et al. 2005), including genes encoding proteins for biosynthesis of the defense hormones JA and Et (Moscatiello et al. 2006; Qutob et al. 2006). Importantly, similarly to our studies, considerable overlap has been found in the responses to different elicitors (Ferrari et al. 2007; Qutob et al. 2006; Thilmony et al. 2006; Zipfel et al. 2006), suggesting that all elicitors activate a conserved basal defense response (Jones and Dangl 2006). However, different experimental conditions (such as tissue type, environmental conditions, duration of treatment, and time of day at harvest) have made it difficult to accurately assess the degree of similarity between responses to diverse MAMPs. In contrast, by simultaneously determining the response to OGs and Flg22 in the seedling assay, we are able to directly compare the responses to these two MAMPs in a tightly controlled experiment.

One intriguing but still unresolved aspect of MAMP signaling is that in contrast to the relatively uniform transcriptional reprogramming observed at early time points following MAMP treatment, the particular suite of early signaling events, or associated kinetics and intensity, vary depending on the specific elicitor (Garcia-Brugger et al. 2006). This variability in response occurs despite the fact that many of the same signaling mechanisms are employed, such as activation of the same MAPK cascades, transient changes in concentrations of nuclear and cytoplasmic Ca^{2+}, activation of kinases and phosphatases, accumulation of reactive oxygen species, and production of NO (Garcia-Brugger et al. 2006). Nühse and colleagues (Nuhse et al. 2000) assayed activation of the Arabidopsis MAP kinase AtMPK6 after exposure of cells or leaf tissue to the elicitors Flg22, OGs, chitin, or xylanase. While all four elicitors lead to rapid and transient activation of AtMPK6, maximal activation occurred at different time points. Similarly, Lecourieux et al (Lecourieux et al. 2005) compared eight different molecules that represent multiple different classes of elicitors (six proteinaceous elicitors, including five that induce necrosis and one, Flg22, that is non-necrotic; and two oligosaccharide elicitors, including OGs) and reported that changes in Ca^{2+} concentration, while induced by all elicitors, varied in

magnitude and timing depending on the stimulus. Our expectation is that these differences in signaling events influence late innate immune responses, either in subtle or obvious ways, to produce a response that is tailored to the specific stimulus.

Roles of Salicylic Acid, Jasmonic Acid, and Ethylene in MAMP Signaling

The relationship between MAMP-mediated defenses and defense-hormone-mediated responses has been unclear. Several lines of evidence suggest that MAMPs stimulate defense pathways that are independent of SA, JA, and Et. Resistance against *P. syringae* or *B. cinerea* can be induced by pre-treatment of Arabidopsis with Flg22 or OGs, respectively, independently of SA, JA, or Et (Ferrari et al. 2007; Zipfel et al. 2004). Consistent with these reports, induction by elicitors of specific defense-related genes has been demonstrated to be independent of SA, JA, or Et (Ferrari et al. 2007; Ferrari et al. 2003; Zhang et al. 2002). Conversely, MAMP elicitors have also been reported to stimulate JA and ethylene production (Doares et al. 1995; Kunze et al. 2004; Simpson et al. 1998), as well as up-regulation of genes encoding proteins involved in the biosynthesis of JA and Et (Moscatiello et al. 2006) or pathogenesis-related proteins linked to SA-mediated responses (Gomez-Gomez et al. 1999). These data suggest that, in addition to innate immune responses that are activated independently of defense hormone signaling, MAMPs may also stimulate defense hormone mediated effects. Figure 1 shows that a variety of genes activated rapidly by OGs and Flg22, identified in the transcriptional profiling studies described above, are activated by OGs independently of *npr1*, *ein2*, and *jar1*, Arabidopsis genes involved in the SA, ET and JA signaling, respectively.

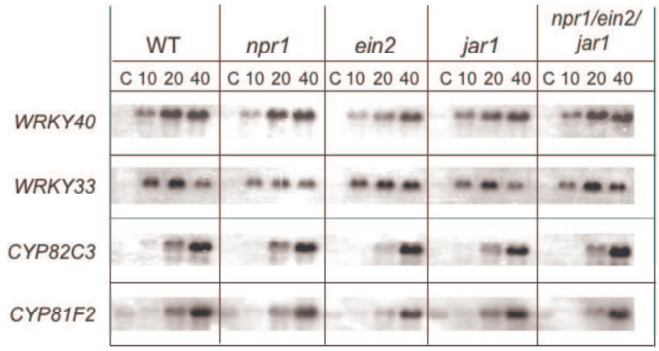

Figure 1. OGs induce early-response genes independently of SA, Et, and JA signaling.

OGs-induced resistance to *B. cinerea* requires functional *PAD3*

OGs released at plant cell walls by the activity of funal polygalacturonases may contribute to resistance through elicitation of specific defense pathways, as pre-treatment of adult plants with OGs reduces the severity of subsequent *B. cinerea* infection (Ferrari et al. 2007). To determine the mechanism of OGs-induced resistance, transcriptional responses in seedlings treated with OGs and in leaves infected with *B. cinerea* were compared. Genes encoding proteins for the biosynthesis of tryptophan-derived secondary metabolites were strongly up-regulated by both challenges (Figure 2), suggesting that this set of metabolites might have a role in OGs-induced resistance. The loss of OGs-induced resistance in *pad3* mutant plants, which are unable to synthesize camalexin, supports the hypothesis that OGs contribute to resistance against *B. cinerea* infection by inducing genes for camalexin biosynthesis (Ferrari et al. 2007).

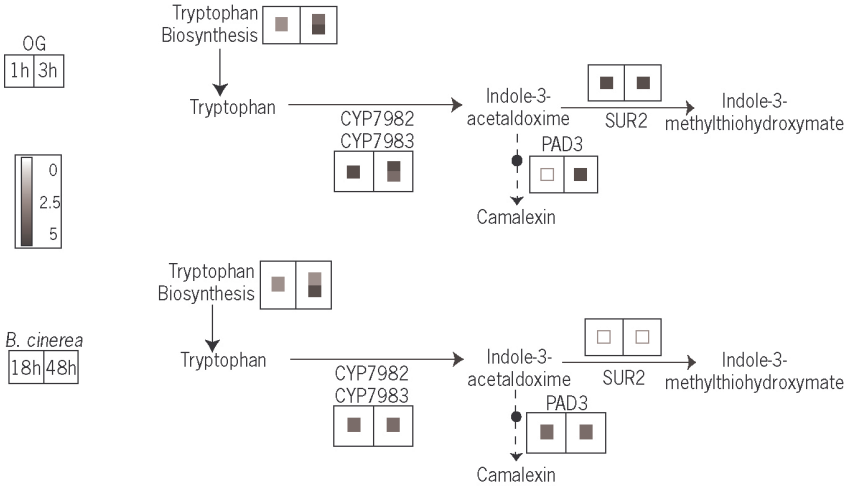

Figure 2. Regulation of tryptophan secondary metabolite genes after elicitation with OGs or infection with *B. cinerea* (Adapted from Ferrari et al., 2007).

Localization of MAMP-induced responses

Little is currently known about localization, either in plant tissues or at a subcellular level, of specific innate immune responses. Seedlings grown in liquid culture have the advantage of representing all vegetative tissue types present in adult plants without requiring the use of either pressure-infiltration or detergents that are often used in spray or dip treatments, both of which can introduce the confounding effects of wounding. Additionally, roots, which are often less-well studied than aerial tissues due to the difficulty in obtaining intact tissue from plants grown on solid medium, are easily assayable. Using this system, we have identified genes that show either root-specific or shoot-specific induction after MAMP treatment (Figure 3), as well as genes that are expressed ubiquitously. Variations of the seedling growth protocol, in which only leaves or only roots are exposed to MAMPs, facilitate our on-going study of defense-related communication between organs.

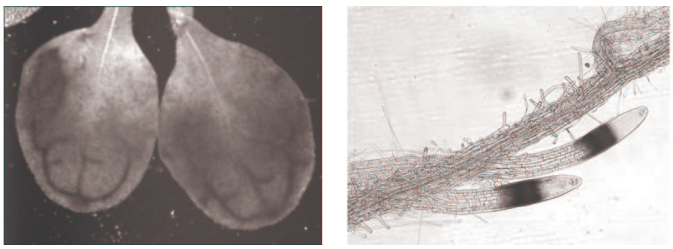

Figure 3. Leaf-specific expression (left) and root-specific expression (right) of two Flg22-induced genes.

Acknowledgements

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ANNEX 3

Activation of Defense Response Pathways by OGs and Flg22 Elicitors in Arabidopsis Seedlings

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Abstract

Pathogen attacks are perceived through recognition of microbe- associated molecular patterns (MAMPs), but the similarities/differences between different MAMP signaling pathways is poorly understood. Transcript profiling of seedlings treated with oligogalacturonides (OGs), plant cell wall-derived elicitors, or the synthetic 22 amino acid polypeptide Flg22, derived from the bacterial flagellin, revealed a transient activation of many genes, including cytochromes P450 *CYP81F2* and *CYP82C3*. Monitoring the expression of these genes in a variety of defense-related mutants suggested that their rapid induction by MAMPs is independent of the salicylicate (SA), jasmonate (JA), or ethylene signaling pathways. Comparison of OGs- and Flg22-elicited genes showed significant overlap and activation of multiple defense signaling pathways, particularly JA-associated processes. However, Flg22 elicited more genes and the amplitude of activation was higher. The magnitude of gene induction was in both cases dose-dependent, but, even at very high concentrations, OGs did not induce as many genes as Flg22. Activation of senescence processes, SA-dependent secretory pathway genes, and *PR1* expression was only observed with Flg22 elicitation.

Keywords: oligogalacturonides, flagellin, Arabidopsis, immune response, transcript profiling, cytochrome P450.

Résumé

Une attaque par des microorganismes, est reconnue par la perception de “microbe-associated molecular patterns” (MAMPs), mais les similarités et différences entre les voies de signalisation qu’ils induisent restent mal connues. Le transcriptome de plantules traitées par les oligogalacturonides (OGs), éliciteurs dérivés de la paroi des plantes, où par Flg22, un peptide de 22 amino-acides du flagelle bactérien, révèle une activation transitoire de nombreux gènes, en particulier ceux codant pour deux cytochromes P450 *CYP81F2* et *CYP82C3*. L’expression de ces gènes dans divers mutants suggère que leur rapide induction par les MAMPs est indépendante des voies de signalisation du salicylate (SA), du jasmonate (JA) ou de l’éthylène. La comparaison des gènes élicités par les OGs et Flg22 montre une corrélation dans l’activation de multiples voies de signalisation, en particulier associées au JA. Flg22 induit une activation plus forte et d’un plus grand nombre de gènes. L’amplitude de l’activation est dose-dépendante dans les deux cas, mais, même aux plus fortes concentrations, les OGs n’induisent pas autant de gènes que Flg22. Seul Flg22 induit une activation de la sénescence, des voies de sécrétion SA-dépendante et de l’expression de la protéine PR1.

Mot-clé: oligogalacturonides, flagelline, Arabidopsis, réponse immunitaire, analyse du transcriptome, cytochrome P450.

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