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Characterization of the oligogalacturonides receptor WAK1 in the defence processes

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1. INTRODUCTION

1.1 Cell wall: the first defensive line

Plants are sessile organisms that rely on a complex, multi-layered innate system to fight pathogen invasion. Protection against pathogen attack occurs initially through a system of passive defence, represented by physical barriers such as the cell wall, the stomata and the cuticle. The cell wall is therefore the first physical barrier that plant cells opposed to pathogens. The cell wall is a dynamic and complex structure composed for the majority of polysaccharides and highly glycosylated proteins with different roles in the physiology and development of the plant (Vorwerk et al., 2004) (Fig. 1.1). In addition to the defence from attack by pathogens, the wall is in fact involved in the structural support, in cell growth and expansion. The first layer to be deposited is the middle lamella, the outermost layer through which adjacent two cells are in contact. At the end of cell division, the two daughter cells lay the next layer, called primary wall. Some specialized cells, during differentiation, lay a further layer, the secondary wall, the structure of which varies depending on the type of cell. The different functions of the cell wall results from the complexly of its structure. It consists of two main components: a microfibrillar component and a matrix. The microfibrillar component consists of microfibrils of cellulose, a

linear polymer of residues of D-glucose linked by glicosidic bonds α (1→4). The matrix surrounding the microfibrils is more complex being constituted by polysaccharides of different nature, proteins and phenolic compounds. The matrix contains hemicelluloses, pectins and proteins (McNeil et al., 1984; Labat-Robert et al., 1990; Carpita and Gibeaut, 1993) (Fig. 1.1).
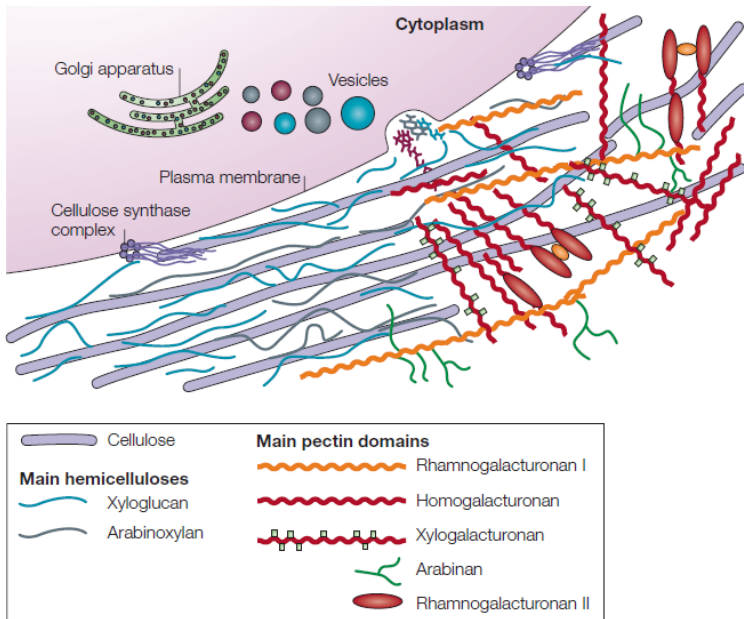
Figure 1.1. The plant cell wall. Cellulose microfibrils (purple rods) are synthesized by large hexameric complexes in the plasma membrane, whereas hemicelluloses and pectins, which compose the matrix polysaccharides, are synthesized in the Golgi apparatus and are deposited to the wall surface by vesicles. For clarity, the hemicelluloses-cellulose network is shown on the left part of the cell wall without pectins, which are emphasized on the right part of the figure (Cosgrove, 2005).

The hemicelluloses are a heterogeneous group of polysaccharides (xylan, glucomannan, mannan, galactomannan and arabinan xiloglucan) that are associated by hydrogen bonds to cellulose microfibrils forming a network. The hemicelluloses, in contrast to cellulose, do not form fibrils as their association is prevented by the presence of side chains and or sequences of monosaccharides not repeated. The pectins are a group of polysaccharides rich in galacturonic acid. The most abundant are homogalacturonan (HGA), rhamnogalacturonan I (RG-I) and rhamnogalacturonan II (RG-II) (Zablackis et al., 1995). HGA is made of repeating galacturonic acid residues that can be *0*-acetylated, methylated or substituted in C-3 with xylose, leading to the formation of xylogalacturonans. HGA is synthesized in the Golgi apparatus, where it is esterified and then secreted in the apoplast, where an enzyme called pectin methylesterase (PME) removes part of the methyl groups. Free carboxylic groups allow the formation of the "egg-box" structures, in which adjacent HGA chains are linked by ionic bonds mediated by calcium ions (Lionetti et al., 2010). (Willats et al., 2001)Other compounds, typical of the secondary wall, are lignin, waxes, cutin and suberin. The lignin is formed after the polymerization by the peroxidase in the presence of H_2O_2 of three aromatic alcohols (sinapilic, coniferilic and cumarilic acids) forming a three-

dimensional network. Lignin has a synergistic action with the cellulose fibrils, conferring resistance to torsion and compression. Waxes and cutin form the cuticle. The waxes are formed by hydrocarbon chains arranged regularly on the surface of the cuticle, or inside it, while cutin is composed by polyesters of fatty acids, oxyacid and epoxy acids. The cutin plays a protective role by forming a hydrophobic layer by avoiding the loss of water. The suberin impregnates the wall, making it hydrophobic and plurilaminated. Its maximum deposition is in the form of cork. It has two functions: limiting the apoplastic flow of solutes and reducing or preventing mechanical attacks of microrganism at the cell wall.

In order to sustain their growth and complete the process of invasion, most of fungi and bacteria secrete cell wall degrading enzymes (CWDE) (Annis and Goodwin, 1997), including exo- and endo-polygalacturonases, pectin lyases and pectate lyases, acetyl esterases, xylanases and a variety of endoglucanases that cleave cellulose, xyloglucan and other glucans (Lebeda et al., 2001).

1.2 Plant immunity

In addition to passive defences, plants employ a multi-layered recognition system to protect themselves against microbial infection. One layer involves generic elicitors, called pathogen

associated molecular patterns (PAMPs) that are recognized by receptor proteins called pattern recognition receptors (PRRs) (Boller and Felix, 2009). PAMPs are typically essential components of whole classes of pathogens, such as bacterial flagellin, bacterial lipopolysaccharides (LPS), peptidoglycans (PGN) or fungal chitin. Plants also respond to endogenous molecules released by pathogen invasion, such as plant peptides or cell wall fragments called damage-associated molecular patterns (DAMPs). Stimulation of PRRs leads to PAMP-triggered immunity (PTI) (Fig. 1.2). The second system of perception involves recognition of race specific elicitors, called effectors. These elicitors are secreted in the apoplast or directly into the cytoplasm of host cell and are recognized by the R proteins, leading to effector-triggered immunity (ETI) (Fig. 1.2). The majority of these R proteins are intracellular receptor proteins of the nucleotide binding-leucine-rich repeat (NB-LRR) type (Dodds and Rathjen, 2010).
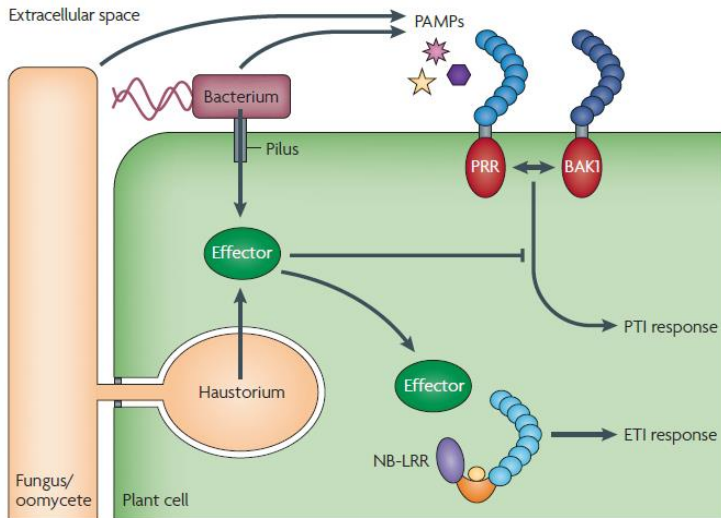
Figure 1.2. Plant Immunity. Recognition of pathogen-associated molecular patterns (such as bacterial flagellin, lipopolysaccharides or fungal chitin) by cell surface pattern recognition receptors (PRRs) triggers PTI (PAMP Triggered Immunity) leading to basal immunity. Many PRRs interact with the related protein BRASSINOSTEROID INSENSITIVE 1-ASSOCIATED KINASE 1 (BAK1) to initiate the PTI signalling pathway. Pathogenic bacteria use the type III secretion system to deliver effector proteins that target multiple host proteins to suppress PTI. Plant resistance proteins (such as NB-LRR) recognize effector activity and restore resistance through effector-triggered immune responses (ETI). Adapted from (Dodds and Rathjen, 2010).

In contrast to PAMPs, effectors are characteristically variable and dispensable. Extreme diversification of ETI receptors and pathogen effectors both within and between species is the norm, whereas some PRR functions are conserved widely across families. Generally, PTI and ETI lead to similar responses including ethylene production, oxidative burst, callose deposition,

9

induction of defence related gene expression, although ETI is qualitatively stronger and faster and often involves a form of localized cell death called hypersensitive response (HR) (Nurnberger et al., 1994). PTI is generally effective against non-adapted pathogens in a phenomenon called non-host resistance, whereas ETI is active against adapted pathogens. However these relationships are not exclusive and depend on the elicitor molecules present in each infection.

PTI and ETI evolved according to the "zig-zag" model proposed by Jones and Dangl (2006), which distinguishes four phases (Fig. 1.3). This model proposes that the first line of active plant defense is formed by pattern recognition receptors (PRRs). In phase 1, PAMPs are recognized by PRRs, resulting in PTI that can halt further colonization. In phase 2, successful pathogens are able to overcome PTI, typically inject effectors directly into the host cytoplasm by type III secretion machinery, resulting in effector-triggered susceptibility (ETS). In phase 3, a given effector is "specifically recognized" by cytoplasmic R proteins, resulting in effector-triggered immunity (ETI). In phase 4, natural selection drives pathogens to avoid ETI either by shedding or diversifying the recognized effector gene, or by acquiring additional effectors that suppress ETI. Natural selection results in new R specificities so that ETI can be triggered again (Jones and Dangl, 2006) (Fig. 1.3).
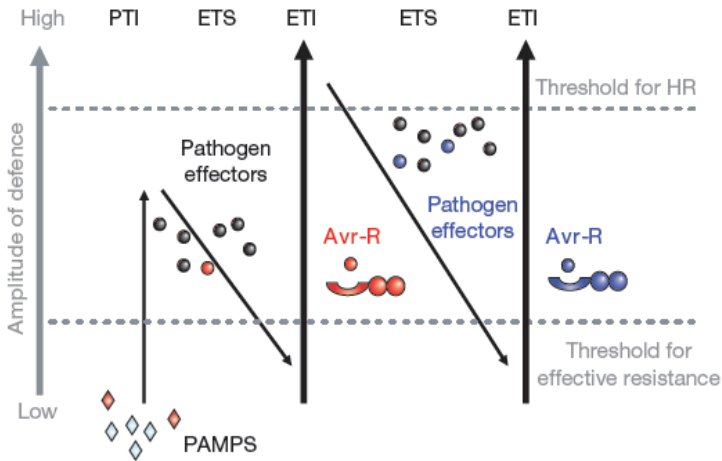
Figure 1.3. The Zig zag model.
This scheme shows the ultimate amplitude of plant disease responses. In phase 1, plants detect pathogen-associated molecular patterns (PAMPs) via PRRs to trigger PAMP-triggered immunity (PTI). In phase 2, successful pathogens deliver effectors that interfere with PTI, resulting in effector-triggered susceptibility (ETS). In phase 3, one effector (indicated in red) is recognized by an NB-LRR protein, activating effector-triggered immunity (ETI), an amplified version of PTI that often passes a threshold for induction of hypersensitive cell death (HR). In phase 4, pathogen isolates are selected that have lost the red effector, and perhaps gained new effectors through horizontal gene flow (in blue) that can help pathogens to suppress ETI. Selection favors new plant NB-LRR alleles that can recognize one of the newly acquired effectors, resulting again in ETI. (Adapted from Jones and Dangl, 2006).

1.3 Basal defence responses after PAMP and DAMP perception

Induction of PTI in response to PAMPs or DAMPs occurs in both host and non-host plant species and is based on basal defence mechanisms. Studies of the effects of PAMPs and DAMPs point to a stereotypical response, indicating that, following PRRs activation, signalling converges to common defence responses.

1.3.1 Very Early Responses (1-5 Minutes):

Ion fluxes. Physiological responses to PAMPs and DAMPs in plant cell cultures start after a lag phase of ~ 0.5–2 min. The first effect is the growth medium alkalinisation, due to changes of ion fluxes across the plasma membrane (Boller, 1995; Nurnberger et al., 2004). It is well known that PAMPs and DAMPs could stimulate an influx of Ca^{2+} from the apoplast leading to a rapid increase in cytoplasmic Ca^{2+} concentrations (Blume et al., 2000; Lecourieux et al., 2002). Ca^{2+} might act as second messenger to determine the opening of other membrane channels, or to activate calcium-dependent protein kinases (Boudsocq et al., 2010).

Oxidative burst. After a lag phase of ~2 min starts oxidative burst (Chinchilla et al., 2007), an immediate and localized reaction that is believed to have several roles in plant defence

(Low and Merida, 1996; Bolwell, 1999). Indeed, reactive oxygen species (ROS) work directly against microbes or may contribute indirectly to defence by causing cell wall crosslinking. Indeed, reactive oxygen species induce the rapid peroxidase-mediated oxidative cross-linking of cell wall lignins, proteins, and carbohydrates, reinforcing the cell wall against enzymatic degradation by pathogens (Cote and Hahn, 1994). Moreover ROS may act as secondary stress signals inducing various defence responses (Apel and Hirt, 2004). It has been shown that ROS induce the expression of defence related genes (Lamb and Dixon, 1997) and act as second messengers in other defence responses, such as systemic acquired resistance (SAR) and the HR (Bolwell, 1999). Although there are many potential source of ROS, genetic and biochemical studies using inhibitors of ROS-generating enzymes have shown that two main categories of enzymes are involved in ROS production in response to pathogens: NADPH oxidases and class III cell wall peroxidases (Daudi et al., 2012). NADPH oxidases have been implicated in biotic and abiotic stress responses and development in different plant species and have been studied in detail in *Arabidopsis thaliana* (Torres and Dangl, 2005). Among the members of the 10-gene family of *RBOH* genes encoding homologs of the mammalian NADPH oxidase gp91 phox (Keller et al., 1998; Torres et al., 2002), AtRBOHD (RESPIRATORY BURST OXIDASE HOMOLOGUE D) and AtRBOHF are required for the

13

production of a full oxidative burst in response to avirulent strains of the bacterial and oomycete pathogens *Pseudomonas syringae* and *Hyaloperonospora arabidopsidis*, respectively (Torres et al., 2002). In addition to NADPH oxidases, class III cell wall peroxidases have been shown to be involved in the generation of a elicitor-mediated oxidative burst (Bindschedler et al., 2006; Daudi et al., 2012). The peroxidase-dependent oxidative burst has been described as a three-component system (Bolwell et al., 2002) involving peroxidases, ion fluxes, and provision of a suitable substrate. The natural physiological substrates used by these peroxidases to generate ROS have not yet been identified (O'Brien et al., 2012).

Activation of MAPKs. An early response to PAMP and DAMP signals is the activation of Mitogen-Activated Protein Kinase (MAPK) cascades (Pedley and Martin, 2005). MAPK cascades are highly conserved modules in all eukaryotes. In plants, MAPK pathways are involved in the regulation of development, growth, programmed cell death and in responses to several environmental stimuli including cold, heat, reactive oxygen species, UV, drought and pathogen attack (Colcombet and Hirt, 2008). Via a phosphorelay mechanism these cascades, minimally composed of a MAPKKK (MAPK kinase kinase), a MAPKK (MAPK kinase) and a MAPK, link upstream receptors to downstream targets. Activated MAPKs phosphorylate a number of different target

proteins. The majority of targets appear to be transcription factors, but other targets include various protein kinases, phospholipases, and cytoskeletal proteins, all of which effect changes in gene expression and/or physiological responses appropriate to the stimulus in question (Widmann et al., 1999). The best-characterized MAPKs in Arabidopsis are MPK3, MPK4 and MPK6, which are activated by a diversity of stimuli including abiotic stresses, pathogens and oxidative stress.

1.3.2 Early Responses (5-30 Minutes):

Ethylene biosynthesis. After 10 min of treatment with PAMPs, an increase in ACC synthase activity (l-aminocyclopropane-1-carboxylate synthase) has been reveled (Spanu et al., 1994). This event triggers an amplified production of the hormone ethylene, known to have a role as stress hormone in plant.

Receptor endocytosis. It is known that in animals receptor endocytosis extends beyond signal attenuation by depleting ligand-binding sites at the plasma membrane (Murphy et al., 2009). Several plant receptors, such as FLS2 (Robatzek et al., 2006) undergo ligand-induced endocytosis. FLS2-GFP construct, stably expressed in Arabidopsis plants, disappears from its plasma membrane localization and appears in vesicles within ~10–20 min after flg22 stimulation (Robatzek et al., 2006). This endocytosis

may serve to remove and degrade the activated receptor (Robatzek et al., 2006).

Gene activation. Treatment of Arabidopsis plants with the PAMPs flg22 and elf18 caused the induction of almost 1000 genes within 30 min and the down-regulation of approximately 200 genes (Zipfel et al., 2006). Although detected by different receptor, oligogalacturonides (OGs), which are known DAMPs, trigger a fast and transient response that is similar to that induced by flg22. However, the response to flg22 is stronger in both the number of genes differentially expressed and the amplitude of change. The magnitude of induction of individual genes is dose-dependent, in response to both elicitors, but, even at very high concentrations OGs do not induce a response that is as comprehensive as that seen with flg22 (Denoux et al., 2008). Interestingly *FLS2* and *EFR* are included in the induced genes, indicating that one role of early gene induction is a positive feedback to increase PRR perception capabilities (Zipfel et al., 2004).

1.3.3 Late Responses (Hours-Days)

Callose deposition.
Callose-containing cell wall appositions, called papillae, are effective barriers that are induced at the sites of attack during pathogen invasion (Luna et al., 2011). Callose is an amorphous,

16

high-molecular weight β-(1,3)-glucan polymer. Callose deposition is typically triggered by bacterial and fungal PAMPs, such as flagellin, EF-Tu, chitin and chitosan (Brown et al., 1998; Gomez-Gomez et al., 1999; Gomez-Gomez and Boller, 2000; Kunze et al., 2004), about 16 h after treatment. Apart from PAMPs, DAMPs, such as oligogalacturonides, from pathogen- or herbivore-damaged plant tissues can activate callose depositions as well (Ridley et al., 2001).

1.4 Non-self recognition: Pathogen-Associated Molecular Patterns (PAMPs)

The ability to determine self from non-self is critical for plants to mount an effective immune response against potential pathogens. Non-self perception is a key element in the defence against pathogens that is mediated by PAMP recognition. PAMPs include a growing list of microbial molecules: lipooligosaccharides of gram-negative bacteria, bacterial flagellin, bacterial Elongation Factor-Tu (EF-Tu), bacterial cold-shock protein (CSP), glucans and glycoproteins from oomycetes, chitin from fungi cell wall, etc. (Nurnberger et al., 2004; Zipfel and Felix, 2005). Often, these molecules play roles in the fitness of microbes, making the pathogens less likely to evade the detection by simple mutations in these molecules (Zhang and Zhou, 2010).

Some of these PAMPs are only perceived by a narrow range of plant species, whereas others trigger defence responses in many species (Zipfel et al., 2006). For example, flagellin induces responses in plants belonging to many different orders, while perception of bacterial CSP and EF-Tu seems to be restricted to the orders of Solanales and Brassicales, respectively (Zipfel et al., 2006).

1.4.1 Examples of perception of PAMP: FLS2/ flagellin and EFR/Ef-Tu

The best-characterised PAMP in plants is flagellin that constitutes the main building block of eubacterial flagella (Zipfel, 2008). Bacterial flagellin is perceived as a PAMP by leucine-rich repeat (LRR) domains of the receptor FLAGELLIN-SENSING 2 (FLS2) in plants (Gomez-Gomez and Boller, 2000) and of the Toll-like receptor TLR5 in vertebrates (Hayashi et al., 2001). The two receptors recognize highly conserved but different epitopes of flagellin (Smith et al., 2003), indicating that flagellin perception systems in animals and plants have evolved independently (Boller and Felix, 2009). Most plant species recognise a highly conserved 22-amino-acid epitope, flg22, present in the flagellin N-terminus (Felix et al., 1999). Flg22 acts as potent elicitor at subnanomolar concentrations (Felix et al., 1999). In *Arabidopsis*, flg22 induces callose formation, accumulation of the defence protein PR1, and strong inhibition of seedling growth (Gomez-Gomez et al., 1999).

The PRR responsible for flagellin recognition in *Arabidopsis thaliana* is the leucine-rich repeat receptor-like kinase (LRR-RLK) FLAGELLIN-SENSING 2 (FLS2) (Chinchilla et al., 2006). FLS2 is composed of an extracellular LRR (leucine-rich repeat) domain, a transmembrane domain and a Ser/Thr protein kinase domain (Gomez-Gomez and Boller, 2000). Functional FLS2 orthologues have been recently identified in the Solanaceae plants *Nicotiana benthamiana* and tomato and in rice (Hann and Rathjen, 2007; Robatzek et al., 2007; Takai et al., 2008). All of these receptors display high levels of identity to *Arabidopsis* FLS2 at the aminoacid level and also mediate flagellin perception (Schwessinger and Ronald, 2012). After flagellin perception, FLS2 rapidly associates with BAK1 (BRI1-associated receptor kinase 1), another LRR-receptor-like kinase, initiating downstream signaling (Lu et al., 2010) (Fig. 1.4).
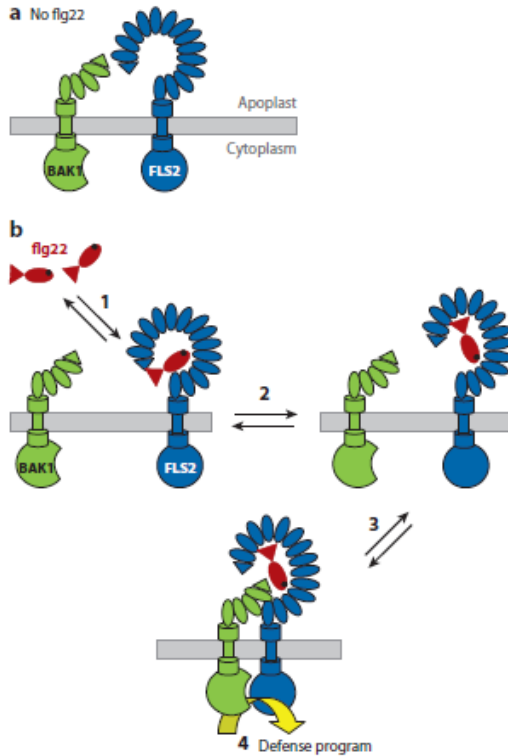
Figure 1.4. Model for the ligand-induced interaction between FLS2 and BAK1. (a) FLS2 and BAK1 do not interact in the absence of flg22. (b) Upon binding of flg22, FLS2 changes its conformation, allowing protein-protein interaction between the extracellular domains of FLS2 and BAK1. This interaction brings the intracellular protein kinase domains of FLS2 and BAK1 in close proximity and initiates signaling, e.g., by transphosphorylation (Boller and Felix, 2009).

Elongation factor Tu (EF-Tu) is the most abundant bacterial protein and is recognized as a PAMP in Arabidopsis and other members of the family Brassicaceae (Kunze et al., 2004). A

20

highly conserved N-acetylated 18 amino acid peptide, elf18, is sufficient to trigger those responses induced by the full-length EF-Tu (Kunze et al., 2004). In Arabidopsis, EF-Tu is recognized by the LRR-RLK EFR (EF-Tu receptor). In contrast to EF-Tu, which is widespread among bacteria, the presence of the EFR seems to be restricted to a small group of plants. This PRR has only been found in members of the Brassicaceae family, indicating that EF-Tu recognition has been acquired only recently during evolution (Kunze et al., 2004). Interestingly, heterologous expression of *A. thaliana* EFR in the non-Brassicaceae plant species *Nicotiana benthamiana* and *Solanum lycopersicum* leads to the ability to recognize EF-Tu, which results in increased resistance to bacterial pathogens (Zipfel et al., 2006; Lacombe et al., 2010). The perception of flg22 and elf18 by FLS2 and EFR, respectively, activates defence responses such as NO$^-$ and ROS accumulation, ion flux, MAPK, callose deposition, ethylene accumulation (Boller and Felix, 2009).

1.5 Self-recognition: Damage-Associated Molecular Patterns (DAMPs)

Response to endogenous signals originating from stressed or injured cells, the so-called "regulation from within," is an important function of the plant immune system (Ferrari et al., 2013). Endogenous elicitors are released from cellular components during pathogen attack or abiotic stresses, and have

21

been indicated as damage-associated molecular patterns (DAMPs). DAMPs typically appear in the apoplast and, similarly to PAMPs, serve as danger signals to activate the immune response (De Lorenzo et al., 2011). A typical example of DAMP is a 18-aminoacid peptide called systemin that triggers a defence response in tomato plants similar to that induced by mechanical wounding (Schilmiller and Howe, 2005). Systemin is derived from a cytoplasmic precursor protein, and is expected to be released only upon cell injury and to act as a DAMP in the neighbouring cells. Receptors that mediate perceptions of systemin have not yet been identified (Hind et al., 2010).

Another example of DAMP is the 23-aminoacid peptide AtPep1 that was isolated from Arabidopsis leaves using an elicitor-induced alkalinization activity assay in Arabidopsis suspension-cultured cells (Huffaker and Ryan, 2007). AtPep1 is derived from the C-terminus of a 92 aa precursor protein AtproPep1, encoded by *PROPEP1*, a gene induced by wounding, cell wall degradation, methyl jasmonate, ethylene, flg22 and AtPep1 (Krol et al., 2010). Constitutive *PROPEP1* overexpression causes an increased resistance against *Pythium irregular* (Huffaker and Ryan, 2007). The receptor of AtPep1 is PEPR1, an LRR-RLK (Shiu and Bleecker, 2001b). Another related LRR-RLK was recently identified, called PEPR2 as a second receptor for AtPep1 (Krol et al., 2010).

1.5.1 An example of DAMP: the OGs.

Oligogalacturonides (OGs) are probably the best characterized plant DAMPs. It has been proposed that OGs are released from plant cell walls upon partial degradation of homogalacturonan, the main component of pectin, by microbial PGs during infections (Cervone et al., 1989). In particular, homogalacturonan is also degraded by the *endo*-polygalacturonases (PGs), the first cell wall hydrolytic enzymes secreted by plant pathogens. The complete hydrolysis of homogalacturonan by fungal PGs is hampered by the apoplastic polygalacturonase-inhibiting proteins (PGIPs); the PG-PGIP interaction favors the accumulation of elicitor-active oligogalacturonides (De Lorenzo et al., 2001). The elicitor activity of OGs is related to their molecular size, being OGs with a degree of polymerization between 10 and 15 the most active elicitors (Cote and Hahn, 1994). This size is optimal for the formation of Ca^{2+}-mediated intermolecular cross-links resulting in structures called "egg boxes" (Braccini and Perez, 2001; Cabrera et al., 2008) that are thought to be necessary for OG activity (Fig. 1.5).
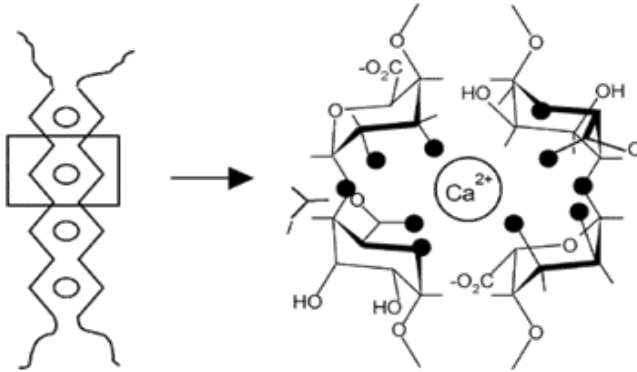
Figure 1.5. Egg boxes formation. Ionic bridges between the carboxyl groups (COO-) of the galacturonic acid residues and calcium ions leads to the formation of intermolecular complexes called "egg-boxes".

OGs elicit in several plant species a wide range of defence responses, including accumulation of phytoalexins (Davis and Currier, 1986), glucanase and chitinase (Davis and Hahlbrock, 1987; Broekaert and Peumans, 1988), deposition of callose, production of reactive oxygen species (ROS; (Bellincampi et al., 2000; Galletti et al., 2008), and nitric oxide (Rasul et al., 2012), (Fig. 1.6).
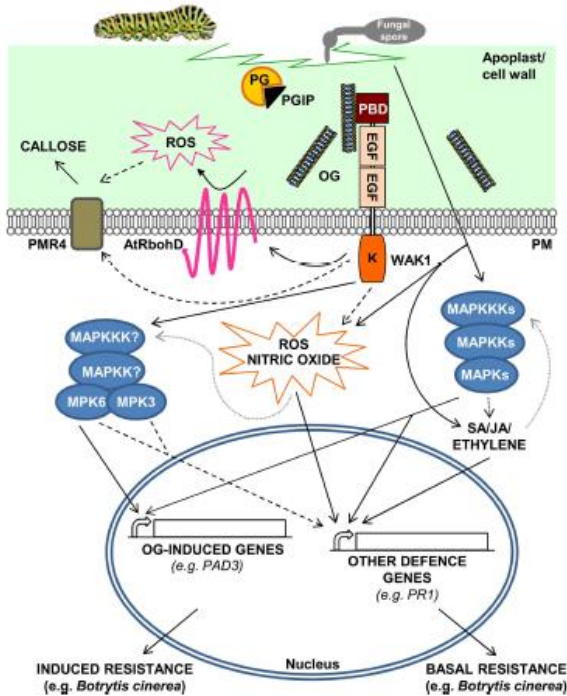
Figure 1.6. Model of defence responses triggered by oligogalacturonides in *Arabidopsis thaliana.* OGs are released from the cell wall after degradation of homogalacturonan by mechanical damage or by the action of hydrolytic enzymes, secreted by pathogens, such as PGs. In the apoplast PGIPs modulate PG activity, favouring the accumulation of elicitor-active OGs. OGs are perceived by the receptor WAK1 (Wall-associated kinase 1) and trigger defence responses such as ROS accumulation through the activation of the NADPH oxidase AtRbohD, nitric oxide production, callose deposition, and MAPK-mediated activation of defence gene expression. Pathogen invasion or mechanical damage also cause an increase of hormones levels (JA, SA, and ethylene), mediated by MAPK cascades, triggering defence responses independently of OGs. DAMP-and hormone-mediated defence responses result, respectively, in induced and basal resistance to necrotrophic pathogens, such as *Botrytis cinerea*. Dashed lines indicate hypothetical cascades; dotted gray lines indicate over simplification of the complex and still partially uncharacterized roles of MAPKs in the regulation of hormone and ROS synthesis/response (Ferrari et al., 2013).

Notably, in Arabidopsis the responses induced by OGs largely overlap those activated by PAMPs. For instance, transcript profiling of seedlings treated with either OGs or flg22 indicates an extensive overlap of responses, at least at the early times after treatment (30-60min; (Denoux et al., 2008)).

In Arabidopsis, flg22 and OGs trigger a set of responses that are independent of ethylene, salicylic acid (SA), and jasmonate signaling (JA) (Zipfel et al., 2004; Ferrari et al., 2007), and induce the activation of two mitogen-activated protein kinases (MAPKs), AtMPK3 and AtMPK6 (Denoux et al., 2008; Galletti et al., 2011). In particular, AtMPK6 seems necessary for the early expression of defence genes and for the induced resistance against the necrotrophic fungus *Botrytis cinerea* triggered by both elicitors (Galletti et al., 2011). Moreover, OGs and flg22 induce a robust oxidative burst mediated by the NADPH oxidase AtRbohD, which is at least partially responsible for the subsequent production of callose (Zhang et al., 2007; Galletti et al., 2008) by the callose synthase POWDERY MILDEW RESISTANT 4 (Nishimura et al., 2003) (Fig. 1.6). However, OGs are relatively weak elicitors compared to flg22, likely because of their reduced half-life (Denoux et al., 2008). For instance, in contrast to OGs, flg22 and other PAMPs, induce also the expression of defence genes dependent on signalling pathways mediated by SA, JA, and ethylene, such as the well characterized SA-dependent marker gene *PR-1* (Denoux et al.,

26

2008). These additional defence responses activated by flg22 likely contribute to basal resistance to pathogens. Furthermore, OGs are endogenous signals likely released in low amounts also in not injured tissues, as a consequence of developmentally related cell wall remodelling processes. Whether plants can distinguish between low physiological doses and higher amounts of OGs produced in pathological situations has not been elucidated yet (Ferrari et al., 2013).

1.5.2 OGs act as signal in the wound response

OGs have been proposed as important signals in the wound response (Bishop and Ryan, 1987; Rojo et al., 1999). Wounding is one of the most common dangers faced by plants, as the injured tissue represents an easy entry point for pathogen. Plants are able to perceive wounded tissues as an altered self and activate localized defences similar to those activated by pathogen infection, such as ROS production (Bradley et al., 1992; Brisson et al., 1994), expression of defence genes (Reymond et al., 2000) and the synthesis of pathogenesis-related proteins (Chang et al., 1995) (Fig. 1.6). Moreover several genes induced by wounding are also regulated in response to pathogens (Reymond and Farmer, 1998; Reymond et al., 2000; Durrant et al., 2000). A study on local and systemic response to wounding in tomato showed that OGs induce proteinase inhibitor (PI) accumulation

(Ryan and Jagendorf, 1995) suggesting a role in the wound response of these DAMPs. OGs can be generated both directly by the physical disruption of homogalacturonan or by the action of wound inducible plant-derived PG, as described in tomato (Bergey and Ryan, 1999). Because of their oligoanionic nature and limited mobility in the tissues, OGs probably act only as local signals (Baydoun and Fry, 1985).

Two separate pathways have been proposed in tomato for the systemic and the local response to wounding: one mediated by the peptide systemin and the hormone jasmonate (JA), responsible for the systemic response, the other mediated by OGs but not by JA, and functioning only locally. The hormone ethylene is required for the full activation of several JA-regulated defense responses (O'Donnell et al., 1996; Ryan and Moura, 2002). It has been proposed a cross-talk between the two pathways because OG-induced oxidative burst in tomato cells is potentiated by systemin (Stennis et al., 1998; Ferrari et al., 2013). In *Arabidopsis*, like in tomato, local and systemic responses to wounding are different (Rojo et al., 1999; Delessert et al., 2004) and OGs up-regulate several wound-responsive genes independently of JA (Leon et al., 2001). However, the wound responses of tomato and *Arabidopsis* considerably differs. For example, genes encoding systemin are absent in *Arabidopsis*. In tomato JA synthesis is induced by OGs and chitosan, whereas JA does not accumulate in *Arabidopsis* plants after treatment with

chitosan. In *Arabidopsis*, chitosan blocks JA-induced gene expression through an ethylene-dependent pathway (Rojo et al., 1999). At present, there is no evidence that OGs induce ethylene synthesis (Ferrari et al., 2008; Brutus et al., 2010) and it is not known whether they block JA-induced responses (Ferrari et al., 2013). Oligogalacturonides induce protection in Arabidopsis and grapevine against *Botrytis cinerea* (Aziz et al., 2004; Ferrari et al., 2007). Notably, a strong resistance against the same pathogen is induced in Arabidopsis after mechanical damage (Chassot et al., 2008). Local resistance induced by both OGs and wounding is independent of SA-, JA-, and ethylene- mediated signalling (Ferrari et al., 2007; Chassot et al., 2007). It has therefore hypothesized that OGs mediate wounding-induced resistance to *Botrytis cinerea* (Ferrari et al., 2013). However, systemic protection against *Botrytis cinerea* observed after treatment with OGs (Ferrari et al., 2007) is not induced after wounding (Chassot et al., 2008). This is probably because the amount of infiltrated OGs is higher than that released in the tissue during mechanical damage (Ferrari et al., 2013). Supporting the hypothesis that OGs mediate at least some responses induced by wounding it has been observed that both OGs (Branca et al., 1988; Bellincampi et al., 1996; Ferrari et al., 2008; Savatin et al., 2011) and wounding (Cheong et al., 2002) repress auxin responses.

1.5.3 OGs in plant growth and development

Pectin is among the first components that are modified when the wall undergoes physiological remodelling. Therefore OGs may be important not only in defence against pathogens, but also under physiological conditions in plant growth and development. Indeed, it has been reported that OGs regulate several developmental-related processes. Most of the developmental effects of OGs may be explained with their ability to antagonize auxin responses, although OGs do not simply act by inhibiting the action of this hormone (Spiro et al., 2002). Auxins, and in particular indole-3-acetic acid (IAA), are crucial for plant growth and development (Leyser, 2002). The first evidence that OGs can antagonize physiological responses to auxins was furnished by Branca et al.(1988), who showed that OGs inhibit competitively auxin-induced elongation in pea stem. Subsequently it has been shown that auxin-induced root formation in tobacco and Arabidopsis leaf explants as well as in thin cell-layer explants is inhibited by OGs (Bellincampi et al., 1993; Savatin et al., 2011). Furthermore, OGs inhibit the stimulation by auxin of the mitotic activity that leads to stomata formation and enhance mean wall thickness of foliar pericycle cells (Altamura et al., 1998). At the molecular level, OGs inhibit the expression of promoters up-regulated by auxin, such as p*rolB* of *Agrobacterium rhizogenes* expressed in tobacco plants (Bellincampi et al., 1996), and

p*Nt114* of tobacco (Mauro et al., 2002). In Arabidopsis OGs inhibit the transcription of auxin-induced genes (*IAA5*, *SAUR16* and *SAUR-AC1*) as well as the activation of the synthetic promoter DR5 (Ulmasov et al., 1997; De Lorenzo et al., 2011). Conversely, auxin inhibits the OG-related protection against *B. cinerea* (De Lorenzo et al., 2011). OG-auxin antagonism is independent of AtrbohD-mediated H_2O_2 accumulation and hormones such as SA, JA or ET (Savatin et al., 2011). Notably, antagonism is not mediated by the microRNA393, which was proposed to mediate inhibition of auxin responses by flg22 treatments (Navarro et al., 2006), nor requires post-transcriptional gene silencing (Savatin et al., 2011). Moreover, OG-auxin antagonism also occurs when the auxin-regulated genes are induced by the translation inhibitor cycloheximide, suggesting that OGs may act downstream of Aux/IAA repressors, possibly at the level of the promoter regions of auxin-responsive genes (Ferrari et al., 2013) (Fig. 1.7).
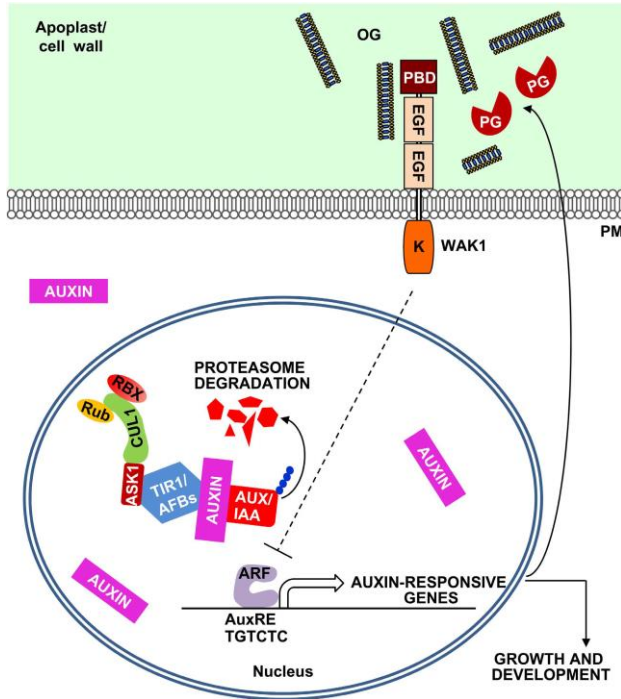
Figure 1.7. A model for the OG-mediated negative feedback regulation of the auxin responses. Plant cells sense auxin through the receptors TIR1/AFBs, F-box proteins that form a SCF E3 ubiquitin ligase complex together with SKP (ASK1) and CULLIN1 (CUL1). This complex is regulated by RUB1 conjugating enzyme (Rub) and RING BOX1(RBX) proteins and, in the presence of auxin, leads to the ubiquitination of Aux/IAA repressors and their proteasome-mediated degradation. Aux/IAA degradation releases auxin response factors (ARFs) that initiate the transcription of auxin-responsive genes, characterized by the presence of auxin response elements (AuxREs) in their promoters. Auxin also induces the expression of plant PGs and other pectin-degrading enzymes (Laskowski et al., 2006). The action of these enzymes may release in the apoplast OGs that can inhibit auxin-related responses, establishing a negative feedback loop (Ferrari et al., 2013).

32

1.6 The pectin integrity monitoring system

Plant cell wall integrity may be efficiently watched by monitoring the pectin status, during an attempted pathogen invasion or when the wall undergoes a stress rupture (De Lorenzo and Ferrari, 2002; De Lorenzo et al., 2011). It has been proposed the existence of a system, called "pectin integrity monitoring system" or PIMS, dedicated to monitor critical structures in the pectin network and alert the cell in the case of danger (De Lorenzo et al., 2011). The biological activity of OGs suggests that they are located in a key position in PIMS, as indicators of cell wall integrity, both in adverse conditions and during normal growth (De Lorenzo et al., 2011; Ferrari et al., 2013). Moreover, a strong and constitutive activation of defences is observed in plants with altered pectin structure (De Lorenzo et al., 2011). This occurs when genes encoding enzymes involved in pectin biosynthesis, such as QUASIMODO 2 or TUMOROUS SHOOT DEVELOPMENT 2, are mutated (Krupkova et al., 2007; Durand et al., 2009), or when exogenous proteins affecting pectin structure are expressed in transgenic plants (Capodicasa et al., 2004; Ferrari et al., 2008). On the other hand, PIMS is not altered by modifications affecting the methylation status of pectin (De Lorenzo et al., 2011). Indeed transgenic plants expressing inhibitors of pectin methyl esterases (PMEIs) (Lionetti et al., 2007) or KO mutants of pectin methylesterase 3 (*pme3*) (Lionetti

33

et al., 2010; Raiola et al., 2011) do not show activation of defence genes as a consequence of the modification. Moreover, because homogalacturonan-degrading enzymes such as PGs are among the first enzymes secreted by microbes during host colonization, PIMS also includes the inhibitors of fungal and insect PGs (PG- inhibiting proteins or PGIPs), which guard the cell wall by limiting degradation of homogalcturonan (De Lorenzo et al., 2001; De Lorenzo and Ferrari, 2002; Di Matteo et al., 2006; Ferrari et al., 2013). By inhibiting the action of PGs secreted by pathogens, PGIPs not only hinder degradation of pectin, but also favor the accumulation of elicitor-active OGs (De Lorenzo et al., 2001; De Lorenzo and Ferrari, 2002) thus playing a dual role in PIMS.

1.7 The Arabidopsis Wall Associated Kinase 1 (WAK1) is a receptor of oligogalacturonides

1.7.1 Wall associated kinases (WAKs)

The identification of an OG receptor has been difficult for a long time. Wall-associated kinases (WAKs) were proposed as interesting candidates because of their ability to bind OGs and polygalacturonic acid (Anderson et al., 2001; Decreux and Messiaen, 2005). WAKs were identified in *Arabidopsis* as pectin-bound proteins, since only harsh treatments, i.e., boiling in the

presence of high concentrations of detergents and reducing agents or pectinase digestion could solubilize a protein reacting with an anti-WAK polyclonal antibody (He et al., 1996; Wagner and Kohorn, 2001; Lally et al., 2001). WAKs are receptor like kinases (RLKs) and consist of an extracellular domain, a transmembrane domain, and a cytoplasmic Ser/Thr kinase domain (Shiu and Bleecker, 2001a). There are five highly conserved *WAK* genes in Arabidopsis (Fig. 1.8) all clustered in chromosome 1 and additional 21 WAK-like genes (Verica et al., 2003). The extracellular domains of the WAKs are 40% to 60% identical to each other and contain two epidermal growth factor-like repeats (Decreux and Messiaen, 2005). In the extracellular portion, while WAK1 and WAK2 share a pectin binding domain (Decreux and Messiaen, 2005), WAK3, WAK4 and WAK5 present subdomains that share some homology with proteins found in the extracellular matrix of mammalian cells (Anderson et al., 2001). The intracellular kinase domains of WAKs are more highly conserved than their extracellular domains (86% of AA identity), which might reflect similar downstream targets; alternatively, this catalytic domain may be more evolutionarily constrained (He et al., 1999).
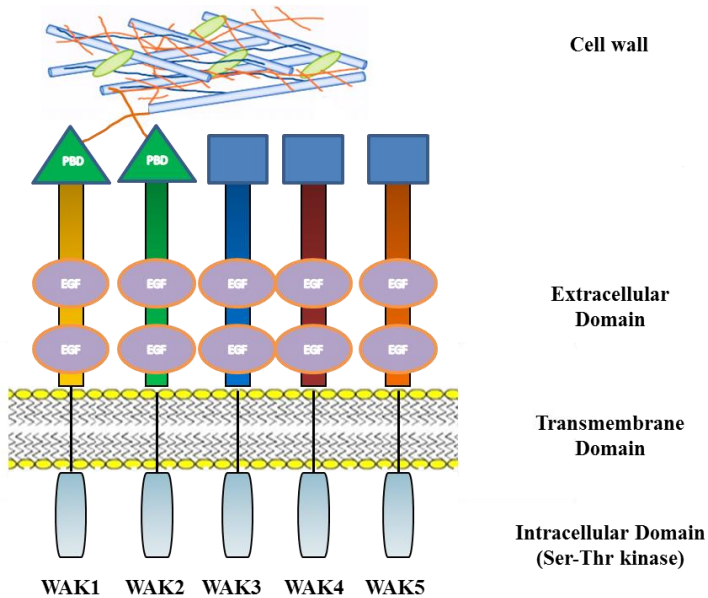
Figure 1.8. The wall-associated kinases (WAKs) in Arabidopsis
WAKs are receptor like kinases (RLK), showing an extracellular domain, a transmembrane domain, and a cytoplasmic Ser-Thr kinase domain. While WAK1 and WAK2 have a PBD (green triangle) in the extracellular domain, WAK 3,4 and 5 show in the extracellular domain a conserved region that share homology with subdomain motifs found in the animal extracellular matrix protein (Anderson et al., 2001). EGF: Epidermal growth factor-like domain.

Several studies have revealed distinct, but overlapping patterns of expression of the five members of the WAKs family. In particular, WAK1 and WAK2 are expressed in stems, expanding leaves and sepals, in shoot and root apical meristems, at organ junctions and, at a lower extent, in flowers and siliques. WAK3 and WAK5 are also expressed in leaves and stems, while WAK4 expression was detected only in siliques. WAKs were not

36

significantly expressed in the elongation zone of roots, the inflorescence stem, cauline leaves, and flower organs other than the base, sepals and ovaries (He et al., 1999; Wagner and Kohorn, 2001). Expression of the WAK genes suggests that most of them play a role in defence. WAK1 and WAK2 are induced by wounding, *P. syringae* infection and aluminum treatment, whereas WAK1, WAK2, WAK3 and WAK5 are all induced by SA (Wagner and Kohorn, 2001; Sivaguru et al., 2003). Public microarray data also indicate that *WAK1*, *WAK2*, and *WAK3* are all induced by *Phytophthora parasitica*, ozone and benzothiadiazole, an activator of the systemic acquired resistance. Interestingly, *WAK1* is induced in *Arabidopsis* seedlings by OGs, but none of the *WAKs* is up-regulated by flg22 (Denoux et al., 2008). Moreover overexpression of *WAK1* confers increased resistance to *Botrytis cinerea* and *Magnaporthe oryzae*, in *Arabidopsis* and rice, respectively (Li et al., 2008; Brutus et al., 2010). WAK proteins probably have a role also under physiological conditions. Since pectin is tightly linked to the extracellular domain of WAKs, these proteins likely act monitoring pectin integrity. Indeed, reduced expression of WAKs, through inducible antisense constructs, causes reduced growth, indicating a role of these proteins in regulating cell expansion (Wagner and Kohorn, 2001; Lally et al., 2001). Moreover a *WAK2* null allele, *wak2-1*, causes a loss of cell

expansion in roots, but only under limiting sugar and salt conditions (Kohorn et al., 2006).

In Arabidopsis genome, it has been found a closely related family of at least 21 WAK like (WAKL) genes (Verica et al., 2003). The predicted WAKL proteins are highly similar in their cytoplasmic region, but are more divergent in their predicted extracellular ligand-binding region. Comparison of the WAKL and WAK gene clusters suggests that they arose independently. Histochemical analyses of WAKL promoters fused with the β-glucuronidase reporter gene have shown that the expressions of WAKL members are developmentally regulated and tissue specific (Verica et al., 2003). In particular, WAKL genes are highly expressed in roots and flowers (Verica et al., 2003). The expansion and size of this family indicates their importance, and some reports have suggested they play a role in pathogen resistance (Verica and He, 2002). Indeed, the expression of WAKL5 and WAKL7 can be induced by wounding stress and by the salicylic acid (SA) analog, 2,6-dichloroisonicotinic acid. Moreover it has been demonstrated that WAKL22 confers resistance to a broad spectrum of Fusarium races (Diener and Ausubel, 2005). Recently, it has been identified a *WAKL10* gene, that is co-expressed with genes that have well defined functional roles in early pathogen defence responses and is induced in response to a range of pathogens and their elicitors (Meier et al., 2010). Moreover the intracellular domain of WAKL10 has a

38

guanylyl ciclase (GC) activity, suggesting that this protein has a functional role in early defence responses and may be, at least in part, responsible for the generation of cGMP, a second messenger involved in biotic stress response in plant (Meier et al., 2010).

Immunoblot analysis with a WAK antibody revealed immunologically related proteins in pea (*Pisum sativum*), tobacco (*Nicotiana tabacum*) and maize (*Zea mays*) (He et al., 1996). Genome sequences analysis revealed that WAK and WAKL family are also present in rice (*Oryza sativa*), (Zhang et al., 2005). In particular were indentified 125 OsWAK gene family members in rice (Zhang et al., 2005). Functions of these OsWAKs are yet to be determined. Phylogenetic analyses of OsWAKs and Arabidopsis WAK/WAKLs show that most OsWAKs and Arabidopsis WAK/WAKLs are clustered in distinct species-specific clades, suggesting species-specific expansion in both plants. Further phylogenetic analyses, comparing OsWAKs with barley HvWAKs, indicate that OsWAK expansion was mainly due to its lineage-specific expansion in monocot species (Zhang et al., 2005). Localized gene duplications appear to be the primary genetic event in OsWAK gene family expansion and the 125 OsWAKs, present on all 12 chromosomes, are mostly clustered (Zhang et al., 2005). The ubiquitous distribution of WAKs implies their potential important roles in plant life processes, but the biological roles of WAKs in these species are little known respect to Arabidopsis. In a recent study, it has been identified a

gene, *OsWAK1*, from rice, which has typical conserved domains and structural characteristics of WAKs (Li et al., 2008). The highest identity over the entire amino acid sequence between OsWAK1 and the known WAK gene is only 27.6%. Biochemical analysis showed that OsWAK1 encodes a functional protein kinase and is associated with the cell wall. Overexpression of OsWAK1 mRNA enhances plant resistance to pathogen infection and northern blotting analysis showed that infection of the rice blast fungus, *Magnaporthe orza* significantly induced the OsWAK1 transcripts. OsWAK1 was also induced after treatment by mechanical wounding, SA and MeJA, but not by ABA, indicating that OsWAK1 is involved in plant defense (Li et al., 2008).

1.7.2 WAK1 is an OG receptor

The role of WAKs as receptors of OGs has been difficult to prove by conventional genetic approaches because of functional redundancy. In particular, Arabidopsis KO mutants for individual *WAK* genes do not show significant phenotypic alterations and generation of double or multiple mutants is difficult because the genes are tightly clustered (He et al., 1999). Moreover transgenic plants constitutively expressing WAK1 or WAK2 antisense transcripts could not be obtained, suggesting that loss of WAK function determines lethality (Wagner and Kohorn, 2001) and no phenotypic alterations were shown by plants with inducible

silencing of individual WAK1 and WAK2, using gene-specific antisense transcripts (Wagner and Kohorn, 2001). WAK1, as the others WAKs, is tightly bound to pectin (Wagner and Kohorn, 2001) and contains two consensus sequence patterns for an EGF2-like domain and a calcium-binding EGF-like domain in the extracellular domain (Anderson et al., 2001). Moreover, WAK1 carries a N-terminal pectin binding domain that interacts with non-methylesterified HGA and OGs in a Ca^{2+}-dependent manner (Decreux and Messiaen, 2005). Notably, OGs with a DP > 9 bind reversibly to WAK1 and the binding increases when OGs are present as dimers in a calcium-mediated "egg box" conformation (Cabrera et al., 2008). Moreover, using site-directed mutagenesis, five basic aminoacids have been identified in the WAK1 ectodomain that are involved in the binding to homogalacturonan dimers and multimers (Decreux et al., 2006) (Fig. 1.9).

Figure 1.9. Aminoacid sequence of WAK1. On the extracellular domain there are a pectin binding domain (PBD) and two EGF like domain. In the ectodomain, in bold, are indicated five basic aminoacid involved in the binding to homogalacturonan (Decreux et al., 2006).

Through a chimeric approach it has been demonstrated that WAK1 act as a receptor of OGs (Brutus et al., 2010). Two different EFR based-chimeras, were designed to dissect the functionality of WAK1 (Brutus et al., 2010). The extracellular domain of WAK1 was fused with the kinase portion of EFR, the receptor of elf18 (Zipfel et al., 2006), and the chimeric receptor (named WEG) was able to activate the kinase domain in response to OGs. On the other hand, upon stimulation with elf18, the chimeric receptor EWAK (composed by the EFR ectodomain and the kinase domain of WAK1) activated the typical responses triggered by OGs, after elf18 treatment (Brutus et al., 2010).

A combination of *in vitro* and *in vivo* studies, by two-hybrid, co-immunoprecipitation and gel filtration chromatography experiments showed that WAK1 interacts and form a complex with AtGRP-3, a glycine rich extracellular protein and with the kinase associated protein phosphatase KAPP (Park et al., 2001). The role of GRP3 and KAPP in OG signalling is still unknown.

2. AIM OF THE WORK

An efficient sensing of danger and a rapid activation of the immune system are crucial for the survival of plants. Conserved pathogen/microbe-associated molecular patterns (PAMPs/MAMPs) and endogenous molecular patterns, which are present only when the tissue is infected or damaged (damage-associated molecular patterns or DAMPs), can act as danger signals to activate the plant immune response. These molecules are recognized by surface receptors that are indicated as pattern recognition receptors (PRRs). Oligogalacturonides (OGs) are well known DAMP, released from the plant cell wall during pathogen infection, by the action of pathogen polygalacturonases (PGs) and their plant inhibitors, polygalacturonase-inhibiting proteins (PGIPs). The PG-PGIP interaction favors the release and the accumulation of elicitor-active oligogalacturonides. OGs have long been considered as local signals in the wound response and since they are negatively charged and have a limited mobility, their activity as a wound signal is likely to be restricted to the areas that are close to the wounded tissue. Recently, it has been demonstrated that the Arabidopsis Wall-Associated Kinase 1 (WAK1) is a receptor of OGs. Using Arabidopsis plants overexpressing WAK1 I investigated the role of WAK1 in perception of the OGs and in the regulation of the wound response.

3. MATERIALS AND METHODS

3.1 Materials

Oligogalacturonides with an average degree of polymerization (DP) from 10 to 15 (OGs) were prepared as previously described (Bellincampi et al., 2000). Short OGs were a 3-6mer pool (OG3-6) purchased from Sigma and evaluated to verify the DP by matrix-assisted laser desorption/ionization time-of-flight MS. The active peptides flg22 and elf18 were obtained by EZBiolab Inc. (Carmel IN, USA). Wild type seeds of *Arabidopsis thaliana* ecotypes Columbia-0 (Col-0) were purchased from Lehle Seeds. Col-0 *efr* seeds were kindly provided by Dr. Zipfel (The Sainsbury Laboratory, Norwich, UK).

3.2 Generation of transgenic plants

WAK1 (AGI code: At1G21250) full-length cDNA clone was obtained from Riken BioResource Center (http://www.brc.riken.jp/lab/epd/). *WAK1* was cloned in frame with and upstream of the eGFP (Enhanced Green Fluorescent Protein) coding sequence by using the Multisite Gateway Recombination Cloning Technology (Life Technologies). In particular a pEN-WAK1 entry clone was generated in the pDONR221/Zeo vector (Life Technologies). Multisite recombination was then performed by using the pEN-R2-F-L3

and pEN-R2-C-L3 vectors, which contain the 35S promoter and the eGFP coding sequence, respectively, and pB7m34GW as destination binary vector, which confers phosphinothricin resistance. All Gateway compatible vectors were previously described (Karimi et al., 2002) and obtained from Plant System Biology (Ghent University; http://gateway.psb.ugent.be/).

EFR (AGI code: At5G20480) full-length gene was amplified by PCR from genomic DNA extracted from Col-0 10-day-old seedlings and introduced into the SmaI and PacI restriction sites of pBI121 vector, which confers kanamycin resistance. Primer sequences used to generate the construct are shown in Table 3.1.

Constructs were verified by sequencing (PRIMM; Milano, Italy). Stable transgenic lines were obtained using the standard *Agrobacterium tumefaciens*-mediated gene transfer procedure (floral dip) (Clough and Bent, 1998), using the *A. tumefaciens* GV3101 strain. Independent transgenic lines expressing WAK1-eGFP and EFR were selected based on their phosphinothricin or kanamycin resistance, respectively. Homozygous plants of the T3 generation, with a single transgene insertion, were chosen for experiments.

Table 3.1. Primers sequences used to generate the constructs

Gene	Forward Primer	Reverse Primer
WAK1	GGGGACAAGTTTGTAC AAAAAAGCAGGCTCC ATGAAGGTGCAGGAG GGTTT	GGGGACCACTTTGTACAA GAAAGCTGGGTAGCGGC C AGTTTCAATGTCCA
EFR	GATACCCCGGGATGAA GCTGTCCTTTTCACTT	GATACTTAATTAACTACA TAGTATGCATGTCCG

3.3 Plant growth and treatment

Arabidopsis plants were grown in soil (Einheitserde) at 22°C and 70% relative humidity under a 12-h light/12-h dark cycle (approximately 120 μmol m^{-2} s^{-1}). For elicitor treatments in adult plant, 4-week-old plants were sprayed with H$_2$O, OGs (70 μg/mL), elf18 (100 nM), flg22 (100 nM) and OG3-6 (70 μg/mL). For seedling assays, seeds were surface sterilized and germinated in multiwell plates (approximately 10 seeds/well) containing 0.5X Murashige and Skoog (MS; (Murashige and Skoog, 1962)) medium supplemented with 0.5% sucrose (2 mL/well). Seedlings were grown at 22°C and 70% relative humidity under a 16 h/8 h

49

light/dark cycle (approximately 120 μmol/m^2/s). After 9 days, the medium was adjusted to 1 mL and treatments with OGs (10 and 50 μg/mL, final concentrations) were performed after 24 h.

3.4 Gene expression analysis

Leaves or seedlings were frozen in liquid nitrogen, homogenized with mixer mill MM301 (Retsch) for 2 min at 25 Hz, and total RNA was extracted with ISOL-RNA Lysis Reagent (5- Prime) 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 PCR analysis was performed using a CFX96 Real-Time System (Biorad). One microliter of cDNA (corresponding to 50 ng of total RNA) was amplified in a 20 μl reaction mix containing 1X GoTaq Real-Time PCR System (Promega) and 0.5 μM of each primer. Three technical replicates were performed for each sample and data analysis was done using LinRegPCR software. 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). Primer sequences are shown in Table 3.2. Marker gene analysis was performed from at least 3

independent biological replicates, each composed by 20 seedlings or at least 4 adult leaves from different plants.

Table 3.2. Primer sequences used in gene expression analysis.

Gene	Forward Primer	Reverse Primer
UBQ (At3G62250)	GTTAAGCTCGCTGTTCTTCAGT	TCAAGCTTCAACTCCTTCTTTC
RET-OX (At1g26380)	CGAACCCTAACAACAAAAAC	GACGACACGTAAGAAAGTCC
WRKY40(At1G80840)	GATCCACCGACAAGTGCTTT	AGGGCTGATTTGATCCCTCT
CYP81F2(At5g57220)	AAATGGAGAGAGAGCAACAACACAATG	ATCGCCCATTCCAATGTTAC
RAP2 (At1g78080)	TTATTACCCGGATTCAACGTT	CCGTAAGCGAAACAAGATCC
WR3(At5g50200)	GACCTGCCCACACAAGATCA	TGGAGGCAATATCTAGGGACGC
PGIP2(At5g06870)	GACTAAGCTGGACCAATCTCAC	AAAAGACTAGGGACCTTTCCTG
WAK1 (At1G21250)	ACAGCACTTGTCTCGATTCT	TCTTTACGCTTGCAGCTCAT

3.5 Callose deposition

Analysis of callose deposition was performed as previously described with slight modifications (Brutus et al., 2010). Briefly, leaves from 4-week-old plants were sprayed with elicitors or wounded by forceps. After 24 h about eight leaves from at least four independent plants, for each treatment, were cleared and dehydrated with 100% boiled ethanol. Leaves were fixed in an acetic acid: ethanol (1:3) solution for 2 h, sequentially incubated for 15 min in 75% ethanol, 50% ethanol, and in 150 mM phosphate buffer, pH 8.0, then stained in 150 mM phosphate buffer, pH 8.0, containing 0.01% (w/v) aniline blue for 16 h at 4°C. After staining, leaves were mounted in 50% glycerol and examined by UV epifluorescence microscope (Nikon, Eclipse E200) using 4x or 10x magnification objective. Filter cubes used was the UV filter (ex. 330-380; em. 400) and the excitation was detected using a cooled charge-coupled device CCD camera (DS-Fi1C). Acquisition software is Nis Elements AR (Nikon).

3.6 Measurement of ROS

H_2O_2 generated by seedlings in response to OGs and elf18 (100 μg/mL and 100 nM, respectively) was measured in the incubation medium by a colorimetric assay based on the xylenol orange dye

(o-cresolsulfonephthalein 3′,3″-bis[methylimino] diacetic acid, sodium salt; Sigma), as previously described (Galletti et al., 2008). To determine H_2O_2 concentration, 500 μL of the incubation medium were added to 500 μL of assay reagent (500 mM ammonium ferrous sulfate, 50 mM H_2SO_4, 200 mM xylenol orange, and 200 mM sorbitol). After 45 min of incubation, absorbance of the Fe^{3+}-xylenol orange complex (A560) was detected. 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. ROS measurement assays were performed from three independent biological replicates, each consisting of 40 seedlings.

3.7 *Agrobacterium tumefaciens*-mediated transient expression

For transient expression of eWAK1 in *Arabidopsis thaliana* seedlings (ecotype Col-0) the procedure described in Li et al., 2009 was followed. Liquid cultures of *A. tumefaciens* were inoculated from colonies frozen glycerol stock. After growth at 28°C in 5 mL LB medium (Luria-Bertani liquid medium, Duchefa Biochemie, Haarlem, The Netherlands) with the appropriate antibiotics for 18-24 hr, the saturated culture was diluted the next day into 10 mL fresh YEB medium (5 g/L beef extract, 1 g/L yeast extract, 5 g/L peptone, 5 g/L sucrose, 0.5 g/L

MgCl$_2$) to OD600 = 0.3 and grown for about 7 hours. Bacterial cells were harvested by centrifugation at 6,000 g for 5 min and washed once with 10 mL washing solution containing 10 mM MgCl$_2$ and 100 μM acetosyringone. After centrifugation at 6000 g for another 5 min, the pellet of bacteria cells was resuspended in 1 mL washing solution. In a clean Petri dish (100 × 20 mm), twenty 4-day-old Arabidopsis seedlings were soaked with 10 mL cocultivation medium containing 0.25 × MS pH 6.0, 1% sucrose, 100 μM acetosyringone, 0.005% (v/v; i.e. 50 μL/L) Silwet L-77 and *A. tumefaciens* cells at final density of OD600 = 0.5 (6×10^8 cfu/mL). Cocultivation was carried out in darkness at the same temperature as seedling growth for 36-40 hour before microscopic observation was performed.

3.8 Confocal microscope analysis

Fluorescence analyses were performed using an inverted laser scanning confocal microscope (LSM780 NLO; Carl Zeiss). Imaging of WAK1-eGFP transiently expressed in Col-0 seedlings were performed using the EC PlanNeoFluor 40x (with 1.3 oil DICII) objective and a 488-nm argon laser with an emission filter of 493-538. The Zeiss ZEN confocal software was used for post-acquisition image processing.

3.9 *Pectobacterium carotovorum* infections

Pectobacterium carotovorum subsp. *carotovorum* (strain DSMZ 30169) was obtained by DSMZ GmbH (Braunschweig, Germany). Bacteria were cultivated in LB for 16-18 h at 28°C, 340 rpm. Next bacterial cells were collected by centrifugation (8000 x g for 10 min) and suspended in a 50 mM potassium-phosphate buffer (pH 7.0) at a final $OD_{600} = 0.05$, corresponding to a concentration of 5×10^7 colony forming units mL^{-1}. Four-weeks old Arabidopsis rosette leaves were detached and placed in Petri dishes containing 0.8% plant agar with the petiole embedded in the medium. Two scratches were made on the epidermis of the adaxial surface of each leaf, at the sides of the middle vein, using a sterile needle and 5 μL of the bacterial suspension were placed on each scratch. Plates were covered with transparent plastic film to maintain the humidity and incubated at 22°C with a 12 h photoperiod for 16 h. Lesion size was then determined measuring the diameter of necrotic area. Infections were performed from three independent biological replicates, each consisting of 16 leaves from four different plants.

4. RESULTS

4.1 Overexpression of WAK1 confers a specific response to OGs

Recent studies have demonstrated that WAK1 is a receptor of OGs, through an experimental approach based on the construction of chimeric receptors (Brutus et al., 2010). In order to characterize the role of WAK1 in the OG perception, I used a reverse genetic approach, based on the expression in Arabidopsis plants of a WAK1-eGFP fusion driven by the CaMV 35S promoter. To evaluate the functionality of the 35S::WAK1-eGFP construct and the correct expression of the protein, I performed *Agrobacterium tumefaciens*-mediated transient expression experiments in Arabidopsis seedlings (Li et al., 2009). Cotyledons of transformed seedlings were analyzed by confocal microscopy. The protein fusion WAK1-eGFP was expressed and localized to the periphery of the cell, presumably at the level of the plasma membrane (Fig. 4.1 A). Furthermore, fluorescence was visible in a reticulated structure, likely representing the endoplasmic reticulum, probably due to the transit of the protein in this compartment during transport to the membrane (Fig.4.1 B). Fluorescence was not observed in seedling transformed with an empty Agrobacterium, used as a negative control (Fig. 4.1 C and D).
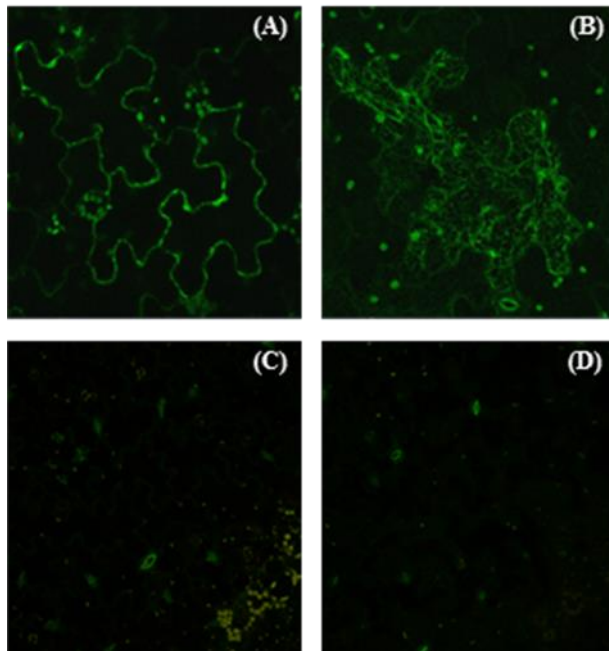
Figure 4.1. 35S::WAK1-eGFP is localized at the periphery of the cell and in the ER. WAK1-eGFP protein fusion was transiently expressed in Arabidopsis seedlings by co-cultivation with *A. tumefaciens* and its expression was analysed by confocal microscopy. Micrographs show WAK1-eGFP localization on the equatorial plane **(A)** and on the cortical plane **(B)**. In panels **C** and **D**, respectively, images show the equatorial and cortical region of Arabidopsis seedlings transformed with a negative control (empty Agrobacterium).

Next, T3 homozygous, single insertion, transgenic Arabidopsis Col-0 plants expressing 35S::WAK1-eGFP (line #4, here on indicated simply as WAK1 plants, and line #2) were obtained. Analysis of *WAK1* transcript by qRT-PCR showed that in WAK1

#2 and #4 seedlings the receptor was overexpressed by 50- and 40- fold, respectively, compared to Col-0 (Fig. 4.2).
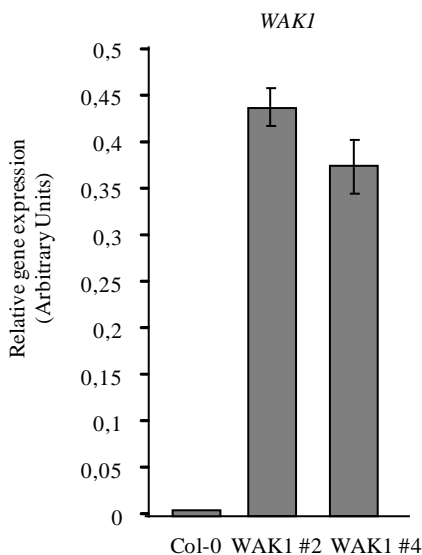

Figure 4.2. *WAK1* transcript levels in WAK1 transgenic seedlings. Analyses were performed by qRT-PCR using *UBQ5* for normalization. Expression of the *WAK1* gene (endogenous + transgene) was evaluated in Col-0 and WAK1 seedlings using primers specific for the region encoding the WAK1 ectodomain.

4.1.1 WAK1 seedlings do not show alteration in the induction of defence gene expression by OGs.

The role of WAK1 in OG perception was investigated by examining the response of the transgenic plants expressing

WAK1-eGFP to OGs in different developmental stages such as seedlings and adult plants. Response to OGs was first examined in seedlings of WAK1 plants by monitoring the expression of genes that are known markers of the response to OGs and PAMPs (Denoux et al., 2008; Galletti et al., 2008). These are *RetOx* (At1g26380), encoding a protein with homology to reticuline oxidases, *WRKY40* (At1g80840), encoding a transcription factor that acts as a negative regulator of basal defence responses, *CYP81F2* (At5g57220), encoding a cytochrome P450 involved in 4-methoxy-indol-3-yl-methyl glucosinolate catabolism (Clay et al., 2009). Ten-day-old WAK1 seedlings were treated with OGs (10 and 50 μg/mL) or water for 1 h, and expression of the marker genes was evaluated by quantitative RT-PCR. WAK1 seedlings exhibited an expression of the three genes after water treatment comparable to that of the wild type (Fig. 4.3). The accumulation of transcripts was also comparable to that of the wild type, after treatment with both concentration of OGs (Fig. 4.3).
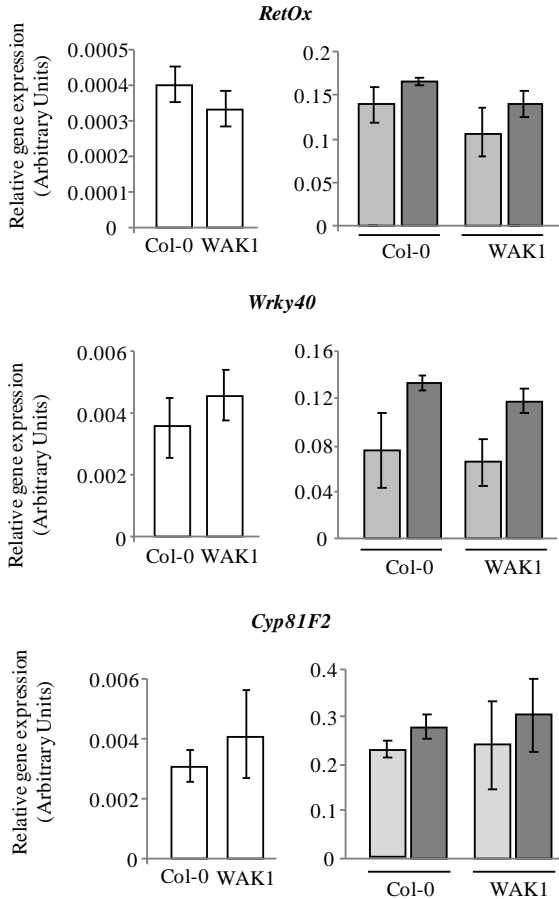
Figure 4.3. Arabidopsis seedlings overexpressing WAK1 do not show alteration in the induction of defence gene expression by OGs. Ten-day-old Col-0 and WAK1 seedlings (line #4) were treated with water (white bar) or OGs (10 and 50 μg/mL, light gray bar and dark gray bar, respectively) and accumulation of *RetOx, WRKY40, CYP81F2* transcripts was analysed after 1 h by Real-Time PCR, using *UBQ5* for normalization. Values are means (± STD) of three independent experiments (n=20, in each experiment). No statistically significant differences between OG treatment of Col-0 and WAK1 seedlings were observed, according to Student's t test.

61

4.1.2 WAK1 seedlings show an H_2O_2 accumulation similar to wild type.

The production of ROS in response to pathogen attack is one of the first measurable events in the plant defence response. It was demonstrated that elicitor treatment induces an extracellular oxidative burst in Arabidopsis (Bailey-Serres and Mittler, 2006; Galletti et al., 2008). Response to OGs in WAK1 seedlings was then investigated by analyzing H_2O_2 accumulation. Fourteen-day-old seedlings were treated with OGs (100 μg/mL) as well as with water or elf18 (100 nM) as controls, and the production of H_2O_2 was determined using an xylenol orange-based colorimetric assay. WAK1 seedlings showed a H_2O_2 accumulation similar to wild type, after treatment with both elicitors (Fig. 4.4 A). The independent transgenic WAK1 line #2 showed a similar behaviour (Fig. 4.4 A). No differences in the production of H_2O_2 between control and WAK1 seedlings were observed using a lower concentration of OG (50 μg/mL) (Fig. 4.4 B).
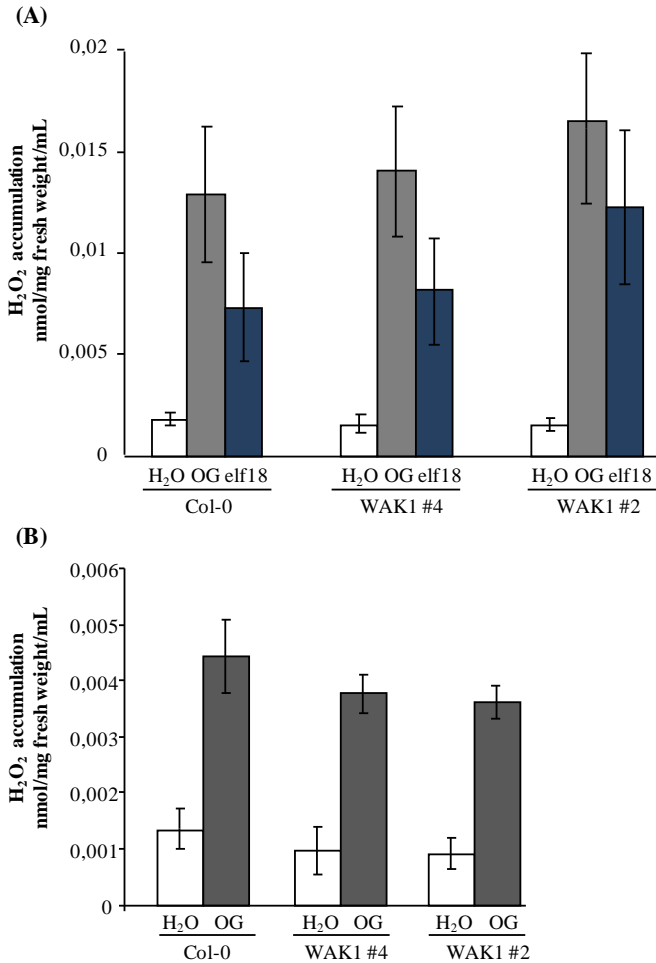
Figure 4.4. WAK1 seedlings show H_2O_2 accumulation similar to wild type.
In panel A, fourteen-day-old WAK1 seedlings (line #4 and #2) were treated with
water (white bar), OGs (100 µg/mL, gray bar) and elf18 (100 nM, blue bar) and
accumulation of H_2O_2 was measured by xylenol orange assay. In panel B,
WAK1 and Col-0 seedlings were treated with water and a lower concentration of
OGs (50 µg/mL, gray bar). Values are means ($\pm$ SE) of three independent
experiments (n=40, in each experiment). No statistically significant differences
between elicitor treatment of Col-0 and WAK1 seedlings were observed,
according to Student's t test.

4.1.3 WAK1 plants show enhanced callose deposition in response to OGs

Response of WAK1 seedlings to OGs did not significantly differ from that of the wild type probably because of a low WAK1 expression level in transgenic seedlings. Indeed, levels of *WAK1* (endogenous + transgene) transcripts, determined by qRT-PCR, were lower in transgenic seedlings than in transgenic rosette leaves (Fig. 4.5), despite the receptor was overexpressed both in transgenic seedlings and adult leaf (40- and 10- fold, respectively) compared to Col-0.
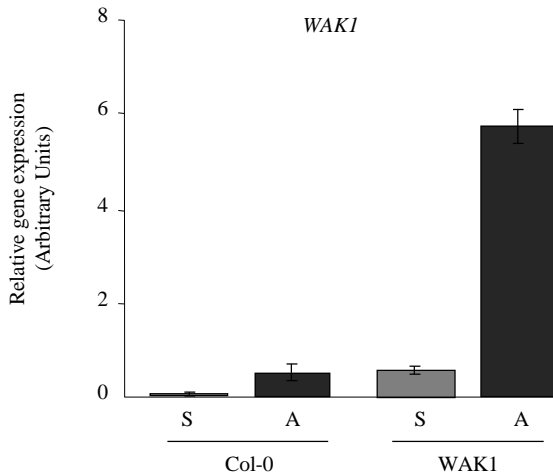

Figure 4.5. **Transgene expression is higher in transgenic rosette leaves than in seedlings.** Analyses were performed by qRT-PCR using *UBQ5* for normalization. Expression of the *WAK1* gene (endogenous + transgene) was

evaluated in seedlings (S) and adult leaves (A) of Col-0 and WAK1 (line #4) plants using primers specific for the region encoding the WAK1 ectodomain.

This result is in agreement with several reports that indicate that the constitutive CaMV 35S promoter has developmental and tissue specificity, as shown by differential GFP expression in tobacco, mustards and cotton (Sunilkumar et al., 2002; Halfhill et al., 2003). Because a difference in response to OGs of WAK1 plants is likely to be better revealed in rosette leaves, due to the higher expression of the receptor, I decided to examine the response to elicitors in leaves of WAK1 plants.

Callose is a β-1,3-glucan deposited at the site of infection and likely acting as a physical barrier against colonization by pathogens. Callose deposition is among the most studied defence responses typically triggered in leaves by PAMPs or DAMPs (Galletti et al., 2008; Luna et al., 2011). The callose deposition response was monitored in WAK1 leaves, as well as in leaves of homozygous Col-0 *efr* plants carrying a single insertion of the CaMV 35S::EFR gene expression cassette (EFR plants), generated during this work and used as controls. EFR plants show a level of *EFR* transcripts 7-fold higher than Col-0 (Fig. 4.6).
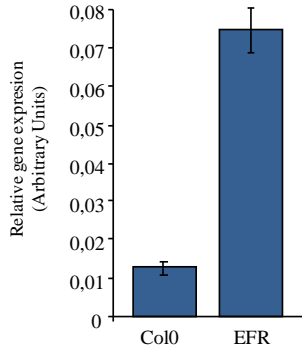
Figure 4.6. *EFR* transcript levels in EFR transgenic plants. Analyses were performed by qRT-PCR using *UBQ5* for normalization. Expression of the *EFR* gene was evaluated in Col-0 and EFR leaves using primers specific for the region encoding the EFR ectodomain

Plants were sprayed with H_2O, OGs (70 µg/mL) or short and biologically inactive OGs (OG3/6, 70 µg/mL) and leaves stained after 24 h with aniline blue for callose visualization. Spray-treatment with OGs induced a very weak response in wild type leaves, but a strong callose accumulation in WAK1 plants (Fig. 4.7), not detectable upon treatment with OG3/6. Enhanced callose deposition in WAK1 leaves occurs specifically in response to treatment with OGs; indeed, treatment with flg22 (100 nM) induced a callose deposition comparable to that of wild type (Fig. 4.7).
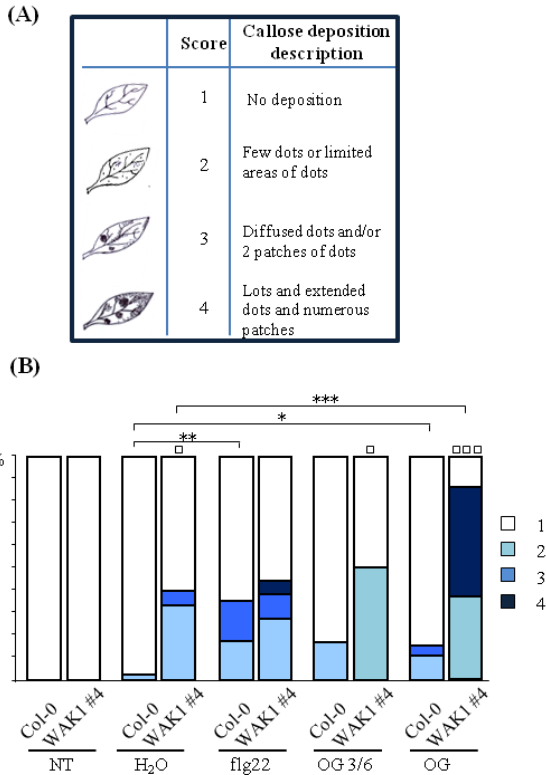
Figure 4.7. Overexpression of WAK1 confers enhanced callose deposition in response to OGs. Leaves were sprayed with H_2O, flg22 (100 nM), short and biologically inactive OGs (OG 3/6, 70 µg/mL) and OG (70 µg/mL), and stained after 24 h with aniline blue for callose visualization. Callose deposition is expressed as a score that varies between 1 (no deposition), 2 (few dots or limited areas of dots), 3 (diffused dots and/or 2 patches of dots) and 4 (lots and extended dots and numerous patches). Representative drawings of callose deposition for each score are shown in panel A. The histograms in panel B show the percentage of leaves with a specific callose deposition score. White squares directly above bars indicate statistically significant difference between Col-0 plants and transgenic plants. Asterisks above connection lines indicate statistically significant difference between water and elicitors treatment in each background plants, according to Fisher's exact test (*or white squares $p < 0,05$; ** $p < 0,005$; *** or white squares $p < 0,0001$). NT: not treated. Five independent experiments were performed (n=12 in each experiments). The second independent WAK1 line (#2) showed a similar behaviour (Fig. 4.9).

The second independent transgenic WAK1 line #2 showed a
behaviour similar to that of the WAK1 plants (line #4) (Fig. 4.8).
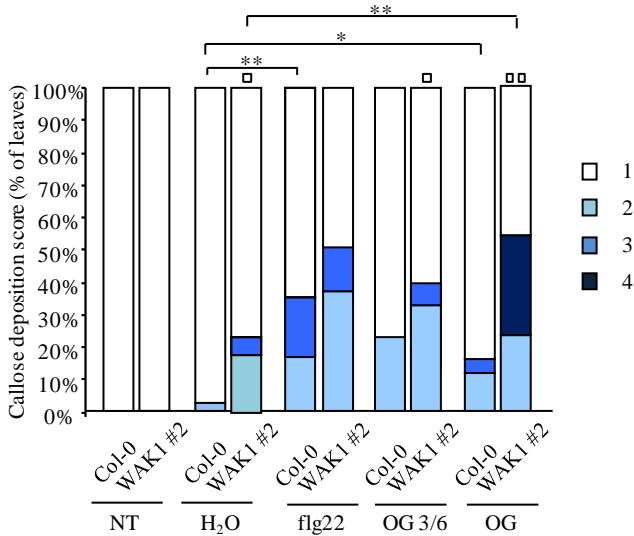
**Figure 4.8. A second transgenic line overexpressing WAK1 show enhanced
callose deposition in response to OGs.** Leaves were sprayed with H_2O, flg22
(100 nM), short and biologically inactive OGs (OG 3/6, 70 µg/mL) and OG (70
µg/mL), and stained after 24 h with aniline blue for callose visualization.
Callose deposition is expressed as a score as described in Fig. 4.7.
Representative drawings of callose deposition for each score are shown in Fig.
4.7 A. The histograms show the percentage of leaves with a specific callose
deposition score. White squares directly above bars indicate statistically
significant difference between Col-0 plants and transgenic plants. Asterisks
above connection lines indicate statistically significant difference between water
and elicitors treatment in each background plants, according to Fisher's exact
test (* or white squares $p < 0.05$; ** or white squares $p < 0.005$; *** $p < 0.0001$).
NT: not treated. Five independent experiments were performed (n=12 in each
experiments).

Conversely, leaves of the EFR plants showed an increased response only to sprayed elf18 (Fig. 4.9), but not to OGs or flg22.
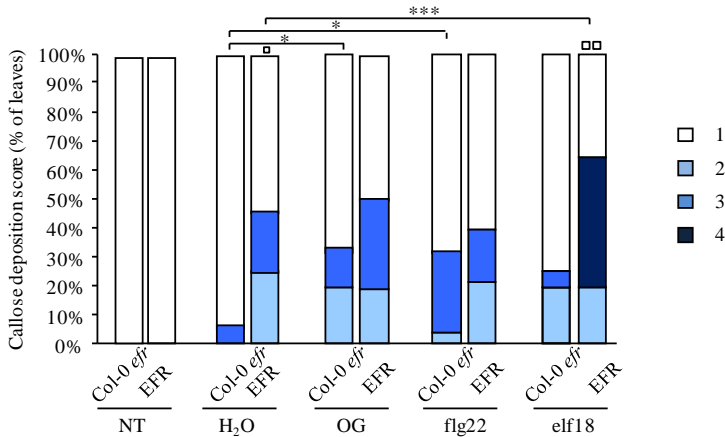
Figure 4.9. Overexpression of EFR confers enhanced callose deposition in response to elf18. Leaves were sprayed with H_2O, OG (70 µg/mL), flg22 (100 nM) and elf18 (100 nM), and stained after 24 h with aniline blue for callose visualization. Callose deposition is expressed as a score as described in Figure 4.7. The histograms show the percentage of leaves with a specific callose deposition score. White squares directly above bars indicate statistically significant difference between control plants (Col-0 *efr*) and transgenic plants. Asterisks above connection lines indicate statistically significant difference between water and elicitors treatment in each background plants, according to Fisher's exact test (*or white squares $p < 0,05$; ** or white squares $p < 0,005$; *** $p < 0,0001$). NT: not treated. Five independent experiments were performed (n=12 in each experiments).

4.2 WAK1 is involved in local response to wounding

OGs have been proposed as important local signals in the wound response (Ryan and Jagendorf, 1995; Leon et al., 2001). OGs are likely to act only as local signals, in areas that are close to the damaged or wounded tissue, because of their oligoanionic nature and limited mobility in the tissues. The availability of WAK1 overexpressing plants, which display an altered response to OGs, gave me the possibility to investigate the role of WAK1 in the wound response.

4.2.1 WAK1 plants show enhanced local callose deposition in response to wounding

To study the involvement of WAK1 in the wound response callose deposition was analysed in leaves in response to mechanical damage inflicted by forceps. Unlike wild type plants, transgenic WAK1 plants showed enhanced callose deposition (Fig. 4.10) in a region surrounding the wound site (i.e. the proximal region) up to a distance of 0.5-1 mm from the wounded site, whereas response at the wound site was comparable to that of wild type leaves. Similar results were obtained using both independent transgenic WAK1 lines.

Figure 4.10. Transgenic WAK1 plants show enhanced local response to wounding. Leaves were wounded by forceps and stained after 24 h with aniline blue for callose visualization. Callose deposition was indicated by different score that varies between 0 (no deposition), 1 (few dots) and 2 (lots dots). Representative callose deposition for each score is shown in panel A; all images are at the same scale. The histograms in panel B show the percentage of leaves with a specific callose deposition score. Experiments were repeated five times (n = 12) with similar results. Asterisks indicate statistically significant difference between control and transgenic plants, according to Fisher's exact test (* $p < 0,0001$).

The response to wounding of transgenic EFR leaves, used as controls, was indistinguishable from that of control leaves (Col-0 *efr*) (Fig. 4.11).

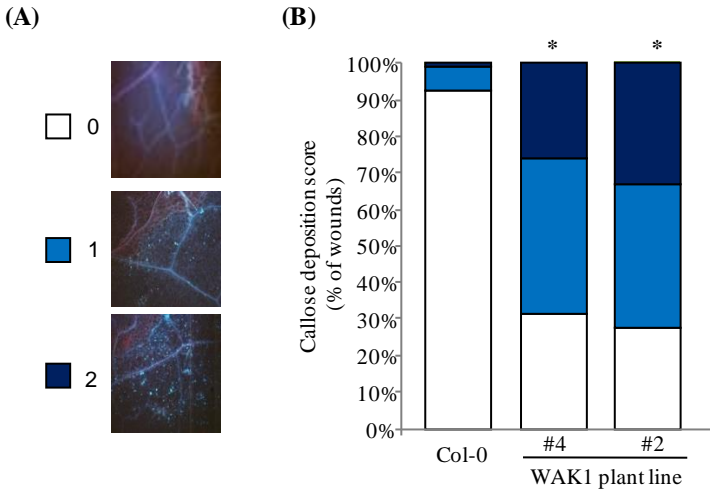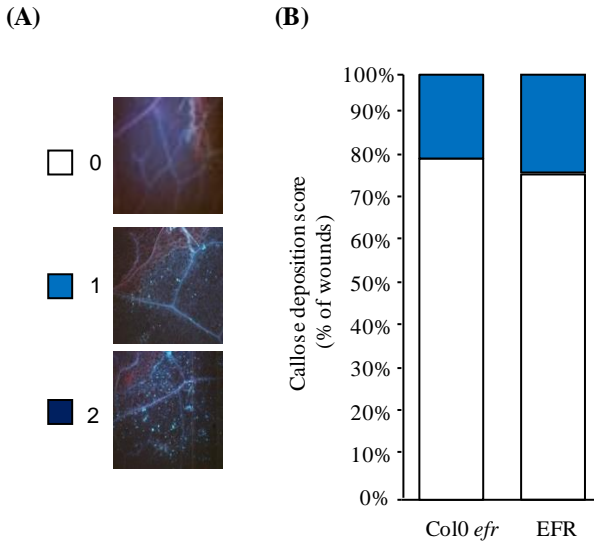**Figure 4.11. Transgenic plants overexpressing EFR do not show enhanced local response to wounding.** Leaves were wounded by forceps and stained after 24 h with aniline blue for callose visualization. Callose deposition was indicated by different score that varies between 0 (no deposition), 1 (few dots) and 2 (lots dots). Representative callose deposition for each score is shown in panel A; all images are at the same scale. The histograms in panel B show the percentage of leaves with a specific callose deposition score. Experiments were repeated three times (n = 12) with similar results.

4.2.2 WAK1 plants show enhanced expression of wounding marker genes

To further investigate the role of WAK1 in the wound response, I analyzed the expression of four wounding marker genes, all expressed locally after wounding: *RAP2* (At1g78080), encoding a AP2 domain-containing protein RAP24 transcription factor

(Delessert et al., 2004) and *PGIP2* (At5g06870), encoding a PG inhibiting proteins (Ferrari et al., 2003); *WR3* (At5g50200), encoding a high affinity nitrate transporter (Titarenko et al., 1997). Adult leaves were wounded by forceps and the expression of wounding marker genes was analyzed in the proximal region, 30 and 60 min after wounding. Mechanical damage caused an increased level of expression in the wound proximal region of WAK1 plants, compared to the wild type, for all the genes analyzed (Fig. 4.12). Basal levels of all genes transcripts in unwounded WAK1 leaves were similar or slightly lower than those of the wild type.
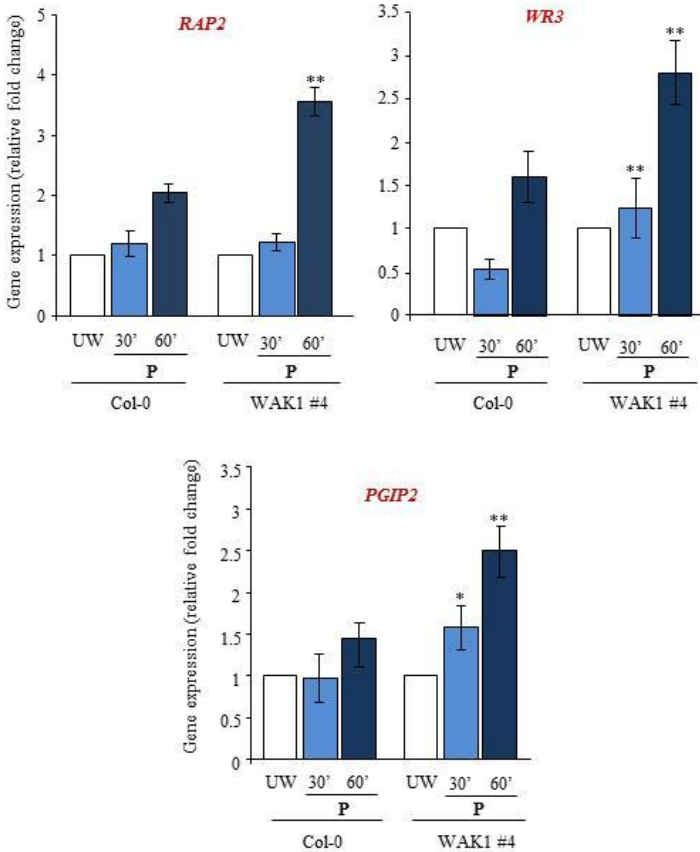
Figure 4.12. WAK1 plants show an enhanced expression of wounding marker genes in the area proximal to the wound site compared to the WT. Leaves were wounded by forceps and the expression of *RAP2*, *WR3* and *PGIP2* was analyzed in unwounded leaves (UW) and in the area proximal to the wound site (P), after 30 (light blue bars) and 60 minutes (blue bars). The fold change relative to unwounded samples was determined by Real-Time PCR using *UBQ5* for normalization. Values are means ± SE of three independent experiments (n=4 in each experiment). Asterisks indicate statistically significant differences between Col-0 and WAK1 (line #4) leaves, according to Student's t test (* $p < 0.01$,** $p < 0.005$).

74

4.3 WAK1 plants show enhanced resistance to *Pectobacterium carotovorum*

In a previous work it has been shown that overexpression of WAK1 confers increased resistance to the necrotrophic fungus *B. cinerea* (Brutus et al., 2010). To better understand the involvement of WAK1 in pathogen resistance, I have investigated the resistance of WAK1 plants toward a necrotrophic bacterium, *Pectobacterium carotovorum* (formerly *Erwinia carotovora)*, the causal agent of black leg and soft rot. Detached leaves of WAK1 (line #4) and Col-0 plants were inoculated with *P. carotovorum*. After 16 hours post infection, WAK1 leaves showed a lesion area reduced by about 50% compared to the control Col-0 leaves (Fig. 4.14), indicating that overexpression of WAK1 confers increased resistance not only to a necrotrophic fungus but also against a necrotrophic bacterial pathogen.
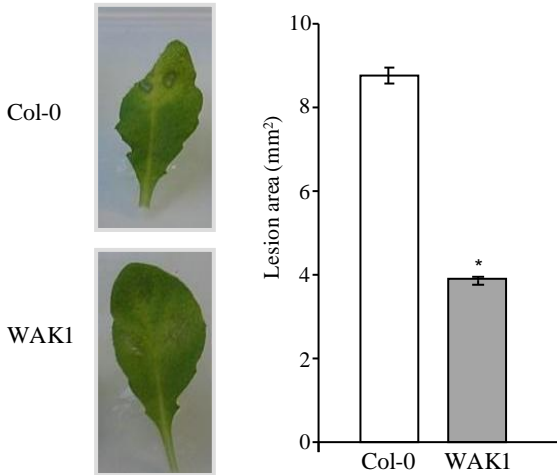
Fig. 4.13. WAK1 plants show enhanced resistance to *Pectobacterium carotovorum*.
Leaves from WAK1 (line #4) and Col-0 plants were inoculated with *P. carotovorum* (5×10^7 CFU/mL) and after 16 hours lesion areas were analyzed. Values are means ± SE of three independent experiments (n=16 in each experiment). Asterisks indicate statistically significant differences against control (Col-0), according to Student's *t* test (* $p < 0.005$).

5. DISCUSSION

Plants are engaged in a continuous co-evolutionary struggle for dominance with their pathogens. The outcomes of these interactions are of particular importance to human activities, as they can have dramatic effects on agricultural systems. Protective mechanisms, collectively referred to as immunity, involve the perception of molecules that alert the cell, known as elicitors. Molecules associated with pathogenic microbes (Pathogen-Associated Molecular Patterns, PAMPs), are specifically sensed by the host cells through Pattern Recognition Receptors (PRRs) and trigger an immune response, known as PAMP-triggered immunity (PTI) (Bittel and Robatzek, 2007; Boller and Felix, 2009). In *Arabidopsis thaliana*, the best-studied PRRs are the leucine-rich repeat receptor like kinases (LRR-RLKs) FLS2 and EFR that specifically bind the bacterial peptides flg22 (derived from flagellin) and elf18 (derived from elongation factor Tu), respectively (Zipfel et al., 2006; Chinchilla et al., 2006). In addition to PAMPs, plant cells recognize molecules from damaged host cells. These damage-associated molecular patterns (DAMPs) are released and recognized upon microbial attack or abiotic stress. A well characterized class of DAMPs is represented by OGs, linear oligomers of α -1,4 D-galacturonic acid residues with a degree of polymerization (DP) ranging from 10 to 15 derived from the non-methylated homogalacturonan of the plant cell wall pectin (Cervone et al., 1989; De Lorenzo et al., 1994; Ridley et al., 2001; De Lorenzo et al., 2011). OGs can be

78

released through the action of pectin degrading enzymes, such as *endo*-polygalacturonases (PGs), which are secreted by pathogenic microbes early during infection. Treatment with OGs induces plant responses that overlap those induced by PAMPs, i.e. accumulation of phytoalexins (Davis et al., 1986), glucanase and chitinase (Davis and Hahlbrock, 1987; Broekaert and Peumans, 1988), deposition of callose, production of reactive oxygen species (ROS, (Bellincampi et al., 2000; Galletti et al., 2008). Moreover treatment with OGs protects grapevine (*Vitis vinifera*) and Arabidopsis leaves against infection with *Botrytis cinerea* (Aziz et al., 2004; Ferrari et al., 2007), suggesting that production of these elicitors at the site of infection contributes to activate defence responses. Pectin is one of the most accessible components of the cell and, therefore, is among the first structures to be altered during an attempted pathogen invasion or when the cell wall undergoes a stress rupture (De Lorenzo and Ferrari, 2002). It has been proposed the existence of a system, called "pectin integrity monitoring system" or PIMS, dedicated to watch the cell wall integrity by monitoring the pectin status (De Lorenzo et al., 2011; Ferrari et al., 2013). OGs are likely located in a key position in PIMS, that allows them to act as indicators of cell wall integrity (Ferrari et al., 2013).

The Arabidopsis wall-associated kinase 1 (WAK1) has been recently identified as an OG receptor (Brutus et al., 2010). WAK1 belongs to a family of five members (WAK1-WAK5), encoded

79

by tightly clustered genes arranged in tandem within 30 kb in chromosome 1 (He et al., 1999). The WAK proteins are RLKs that are tightly bound to the cell wall (He et al., 1996) and consist of an extracellular domain, a transmembrane domain, and a cytoplasmic Ser/Thr protein kinase domain.

Understanding the role of WAK1 in pathogen response is of great importance, not only for a better knowledge of plant physiology, but also to improving crop yield and performance. To study the involvement of WAK1 in the OG perception I used a reverse genetic approach, analyzing the response to OGs in WAK1 transgenic plants. In particular I analyzed well-known defence responses activated by both PAMPs and DAMPs such as the early induction of defence gene expression, H_2O_2 accumulation and callose deposition (Denoux et al., 2008; Galletti et al., 2008; Luna et al., 2011) in different developmental stages, seedlings and adult plants.

The expression of *WAK1* transcript in transgenic plants seems to be developmentally regulated and/or tissue specific. Indeed, even though the receptor results overexpressed both in seedling and adult transgenic plants, levels of *WAK1* transcripts were lower in transgenic seedlings than in transgenic rosette leaves, in agreement with several reports that suggest a developmental and tissue specificity of the expression of the constitutive CaMV 35S promoter (Sunilkumar et al., 2002; Halfhill et al., 2003). Probably because of the low level of transcript in WAK1 seedlings,

80

induction of defence marker genes (*RET-OX, WRKY40, CYP81F2*) and accumulation of H_2O_2 in transgenic seedlings is comparable to that of the wild type. In WAK1 adult plants, in which the transcript level is higher, responsiveness to sprayed OG is also higher in term of callose deposition, in treatment conditions in which the wild type plants show a weak response. A strong callose accumulation is not visualized upon flg22 or short and inactive OG (OG3/6) compared to the wild type. The same enhanced and specific responses were observed in adult EFR transgenic plants, used as control, after elf18 treatment, but not after OG treatment, demonstrating that the enhanced response to OG is specific of WAK1 overexpressing plants.

One of the most common dangers faced by plants is wounding as injured tissue offers an ideal entry point for many microorganisms that may invade plants. Plants have evolved mechanisms to recognize and respond to these injuries by activating various resistance mechanisms against micro-organisms or insects. OGs have been hypothesized to be involved in wound signalling, because they can be generated both directly by the physical disruption of homogalacturonan and by the action of endogenous PGs. Indeed, a tomato PG has been described to be responsible for the production of OGs after wounding (Bergey and Ryan, 1999). However, OGs are likely to act only as local signals, because of their oligoanionic nature and limited mobility in the tissues (Baydoun and Fry, 1985). I have observed that mechanical

81

damage causes an enhanced callose deposition in a region surrounding the wound site (the proximal region) in leaves of WAK1 plants after wounding inflicted by forceps. Moreover mechanical damage caused an increased expression of wounding marker genes, all expressed locally after wounding, i.e. *RAP2, PGIP2*, *WR3* (Titarenko et al., 1997; Ferrari et al., 2003; Delessert et al., 2004) in the wound proximal region of WAK1 plants.

In this study, I have demonstrated that WAK1 can perceive the OGs, and is involved in the wounding perception.

These results indicate WAK1 as the first receptor known to be involved in the response to wounding and support the hypothesis that OGs act as local signal molecules that are accumulated during cell wall degradation due to the wound process.

Recently it has been demonstrated that Arabidopsis plants can respond not only to wounding, but also to a gentle forms of mechanical stimulation (soft mechanical stress) activating defense response against the virulent fungus *B. cinerea* (Benikhlef et al., 2013). It would be interesting to analyze if WAK1 plants show an enhanced response to soft mechanical stress as well as to wounding.

As the wound is an entry point for pathogens that invade the plants, it is also interesting to analyze whether the overexpression of WAK1 confers increased resistance to pathogens. It has already been shown that the WAK1 plants are more resistant to

the necrotrophic fungus *Botrytis cinerea*. In this study I have further demonstrated that overexpression of WAK1 confers enhanced resistance to a necrotroph bacterium, *Pectobacterium carotovorum*.

It would be intriguing to extend these study by analyzing not only resistance to other microbial or fungal pathogens, but also to insect herbivores. Furthermore it could be assessed if these plants exhibit altered basal levels of genes involved in pathogens resistance and analyze the kinetics and the level of induction of these genes following treatment with pathogens.

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