Title: Immune responses induced by oligogalacturonides are differentially affected by AvrPto and loss of BAK1/BKK1 and PEPR1/PEPR2

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Summary

Plants possess an innate immune system capable of restricting invasion by most potential pathogens. At the cell surface, recognition of microbe-associated molecular patterns (MAMPs) and/or damage-associated molecular patterns (DAMPs) by pattern recognition receptors (PRRs) represents the first event for promptly mounting an effective immune response. Pathogens have evolved effectors that block MAMP-triggered immunity. The *Pseudomonas syringae* effector AvrPto abolishes immunity triggered by the peptide MAMPs flg22 and elf18, derived from the bacterial flagellin and Elongation factor Tu, respectively, by inhibiting the kinase function of the corresponding receptors FLS2 and EFR, as well as of their co-receptors BAK1 and BKK1. Oligogalacturonides (OGs), a well-known class of DAMPs, are oligomers of alpha-1,4-linked galacturonosyl residues released upon partial degradation of the plant cell wall homogalacturonan. We show here that AvrPto affects only a subset of the OG-triggered immune responses and that, among these responses, only a subset is affected by the concomitant loss of BAK1 and BKK1. On the other hand, the antagonistic effect on auxin-related responses is not affected by either AvrPto or the loss of BAK1/BKK1. These observations reveal an unprecedented complexity among the MAMP/DAMP response cascades. We also show that the signaling system mediated by Peps, another class of DAMPs, and their receptors PEPRs, contributes to OG-activated immunity. We hypothesize that OGs are sensed through multiple and partially redundant perception/transduction complexes, some targeted by AvrPto but not necessarily comprise BAK1 and BKK1.

INTRODUCTION

Plants, as sessile organisms, are continually exposed to adverse conditions, both biotic and abiotic. Due to the absence of an adaptive immune system, plants rely on an innate immune system that depends on an efficient pathogen sensing and rapidly establishment of defense responses (Gomez-Gomez*,* 2004; Bohm *et al.,* 2014). Pathogen detection occurs through direct binding broadly conserved pathogen molecules (pathogen/microbe-associated molecular patterns, PAMPs/MAMPs) by a large arsenal of trans-membrane pattern-recognition receptors (PRRs). Specific PRRs also detect host-derived damage-associated molecular patterns (DAMPs), including cell wall fragments or peptides produced by mechanical injuries or lytic activities of microbe-secreted enzymes (Savatin *et al.,* 2014b; Benedetti *et al.,* 2015). Activated PRRs trigger immune responses by switching on different and parallel signal transduction pathways. This represents the first layer of the plant immune system and is known as PAMP- (or pattern)-triggered immunity (PTI) (Zipfel*,* 2014). Successful pathogens must overcome PTI, in order to cause disease, either by evading or suppressing this important first layer of plant innate immunity. To do this, pathogens evolved effectors, which are delivered both to the plant apoplast and inside the host cells. In an evolutionary arms race, plants developed the ability to detect such effectors trough other types of receptors called Resistance (R) proteins and to counteract the invasion by activating the effector-triggered immunity (ETI) (Jones and Dangl*,* 2006; Macho and Zipfel*,* 2015).

Studies on plant defense-related signaling pathways have focused on MAMP perception and transduction. So far, the best studied PRRs are FLAGELLIN-SENSITIVE 2 (FLS2) and EF-Tu RECEPTOR (EFR), which bind flg22, an epitope derived from the bacterial flagellin, and elf18, an epitope derived from the elongation factor thermo-unstable, respectively (Gomez-Gomez and Boller*,* 2000; Zipfel *et al.,* 2006). On the other hand, a well-known class of DAMPs is represented by the oligogalacturonides (OGs), which are

pectin-derived oligosaccharides released from plant cell walls upon partial degradation of homogalacturonan (Ferrari *et al.,* 2013). In *Arabidopsis thaliana* WALL-ASSOCIATED KINASE 1, a transmembrane receptor kinase containing epidermal-growth factor-like repeats, was identified as an OG receptor (Brutus *et al.,* 2010). A second well-characterized class of DAMPs is represented by the Arabidopsis peptides Pep1-8, which originate from the cleavage of the corresponding precursor proteins named PROPEPs (Huffaker *et al.,* 2006). Expression of *PROPEP* genes is induced in response to both biotic and abiotic stimuli as well as during development (Huffaker *et al.,* 2006; Bartels *et al.,* 2013). Peps are differentially perceived by PEP1 RECEPTOR 1 (PEPR1), which binds Pep1-6, and PEPR2, which, instead, binds only Pep1 and Pep2 (Krol *et al.,* 2010; Yamaguchi *et al.,* 2010).

Reverse genetic approaches have elucidated elements involved in MAMP signaling, for example the important role of the co-receptor BRI1-ASSOCIATED RECEPTOR KINASE1 (BAK1/SERK3) and its closest paralog BAK1-LIKE1/SERK4 (BKK1/SERK4), both members of SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE (SERK) gene family (Albrecht *et al.,* 2008), in the response to flg22 and elf18 (Kim *et al.,* 2013). BAK1 is also required for response to other MAMPs like HrpZ, peptidoglycan and lipopolysaccharide, but not chitin or NPP1 (Shan *et al.,* 2008), and along with BKK1, mediates responses to Pep1 (Roux *et al.,* 2011).

The current vision is that, after flg22 or elf18 perception, BAK1 and BKK1 form tight complexes with both FLS2 and EFR to initiate downstream signaling (Chinchilla *et al.,* 2007; Roux *et al.,* 2011). BAK1 and BKK1 also provide partially overlapping activity in brassinosteroid (BR)-dependent signaling pathway (He *et al.,* 2007).

AvrPto is a type III kinase inhibitor effector of the hemibiotrophic bacterium *P. syringae* pv *tomato* DC3000 (*Pst*) that triggers gene-for-gene resistance and hypersensitive response (HR) in tomato plants carrying the corresponding *R* gene *Pto*, encoding a serine/threonine

kinase (Pedley and Martin, 2003). In Arabidopsis, AvrPto suppresses plant defense responses elicited by MAMPs (Hauck *et al.,* 2003; Cui *et al.,* 2005; Li *et al.,* 2005). For example, all flg22-induced responses tested so far, i.e. accumulation of H_2O_2 (Xiang *et al.,* 2008), mitogen-activated protein kinases (MAPK) activation and induction of defense related genes (He *et al.,* 2006), are strongly reduced by AvrPto. Moreover, constitutive *in planta* expression of AvrPto affects plant development, leading to a phenotype similar to that of weak mutants insensitive to BRs (Shan *et al.,* 2008). Suppression of MAMP-induced responses by AvrPto occurs through direct binding and inhibition of elements in the perception complexes. A dispute is open as to whether this effector targets BAK1 or FLS2 (Shan *et al.,* 2008; Xiang *et al.,* 2008), and evidence in support of both possibilities has been provided (Goehre *et al.,* 2008; Xiang *et al.,* 2011; Cheng *et al.,* 2011).

Because there is a large overlap between responses elicited by OGs and MAMPs (Asai *et al.,* 2002; Denoux *et al.,* 2008; Galletti *et al.,* 2008; Galletti *et al.,* 2011), it is conceivable that MAMPs and OGs share some elements in their signaling pathways.

In this work, we have investigated whether in Arabidopsis AvrPto inhibits OG-induced immunity and whether BAK1 and BKK1 are involved in OG signaling, using plants that express AvrPto in an estradiol-inducible manner and single and double *bak1-5* and *bkk1-1* mutants. *bak1-5* is a semi-dominant allele carrying a single amino acid substitution in the kinase domain of BAK1 that reduces its activity (Schwessinger *et al.,* 2011). The mutant exhibits normal growth and is strongly impaired in flg22- and elf18-induced responses (Roux *et al.,* 2011; Schwessinger *et al.,* 2011). On the other hand, the *bak1-5 bkk1-1* double mutant, which also has a normal phenotype shows more marked defects in the early and late responses to flg22 and elf18 compared to the *bak1-5* mutant, and is more susceptible to *Pst* and the obligate biotrophic oomycete *Hyaloperonospora arabidopsidis* (Roux *et al.,* 2011).

We show here that, unlike the response to flg22, only a subset of defense responses induced by OGs is suppressed in the presence of AvrPto and, in turn, only a subset of these is affected by loss of both BAK1 and BKK1. Moreover, we show that the Pep-PEPR signaling system contributes to OG-induced resistance to *Botrytis cinerea* and full induction of *PATHOGENESIS-RELATED GENE1* (*PR1*). Induction of *PHOSPHATE-INDUCED 1* (*PHI-1*) and inhibition of auxin-regulated gene appear instead independent of all these elements. Our observations reveal a complexity in the OG signaling pathways that is unique among the characterized MAMPs and DAMPs and suggest that OGs are sensed and transduced by multiple and redundant complexes, only some of which involve BAK1 and BKK1 and are targeted by AvrPto.

RESULTS

AvrPto inhibits OG-triggered ROS production and callose deposition, but partially affects activation of MPK3 and MPK6

Whether AvrPto blocks the signal transduction triggered by OGs was investigated, by generating β-estradiol inducible AvrPto plants and analyzing responses that are typical readouts of the OG action. Seven T1 independent plants showing the presence of *AvrPto* transcripts in excised leaves treated with β-estradiol (1 μM) for 48 hours were initially selected; all were allowed to self-pollinate to produce T2 seeds. For further analysis, two lines (#4 and #5) were chosen that showed a 3:1 segregation of the resistance to the antibiotic hygromycin in the T2 progeny, and homozygous T3 seeds (#4.1 and #5.1) were obtained. Transcript levels, examined in T3 seedlings treated for 48 hours with 1 μM β-estradiol, were higher in line 4.1 than in line 5.1; as expected, AvrPto transcripts were absent in wild-type plants (Fig. S1a). The AvrPto 4.1 line, germinated and grown for ten days in the presence of the inducer (1 μM), displayed an overall inhibition of shoot and root development and a high number of root hairs. The AvrPto 5.1 line, grown in the same

conditions, showed a similar but less pronounced phenotype (Fig. S1b). In these lines, responses to OGs and flg22 were analyzed in seedlings grown in the presence of β-estradiol (1 μM), or dimethyl sulfoxide (DMSO) as a control, for the last 2 days before elicitor treatment, and in adult plants, sprayed with β-estradiol (10 μM) or DMSO, three times within a week, before elicitor treatment or infection.

Both OGs and MAMPs rapidly and transiently activate, through phosphorylation, MAP kinase cascades including the single kinases MPK3 and MPK6 (Zipfel *et al.,* 2006; Galletti *et al.,* 2011; Savatin *et al.,* 2014a). We examined levels of phosphorylated MPK3 and MPK6 in transgenic and wild-type seedlings 5, 10 and 15 minutes after elicitor treatment by western blot analysis, using a commercial antibody generated against the human homologs of these MAPKs (α-p44/p42). In response to OGs, activation of MPK3 and MPK6 was partially reduced only in the high-expressing AvrPto 4.1 line compared to the wild type (Fig. 1), whereas, it was completely abolished and strongly reduced in lines 4.1 and 5.1, respectively, in response to flg22, as expected (He *et al.,* 2006).

Extracellular ROS production is another well-known response to OGs as well as to MAMPs that, in Arabidopsis, is mediated by the NADPH oxidase AtrbohD (Galletti *et al.,* 2008). OG-induced extracellular production of hydrogen peroxide by adult leaf discs of the AvrPto line 4.1 was found severely affected compared to that of the wild type; the defect was confirmed, although less pronounced, in the second independent line, i.e. #5.1 (Fig. 2a). Callose deposition, another typical response to OGs and flg22 that is mediated by RBOHD-dependent hydrogen peroxide production (Zhang *et al.,* 2007; Galletti *et al.,* 2008), was also impaired in both AvrPto expressing lines compared to wild type (Fig. 2b). ROS production and callose accumulation were defective in response to flg22 in both AvrPto lines (Fig. 2a and b), as expected (Xiang *et al.,* 2008).

AvrPto affects OG-regulated expression of a subset of defense response genes

Because transcriptional reprogramming is another response to MAMPs and OGs (Denoux *et al.,* 2008), the effect of AvrPto on OG-triggered defense gene induction was analyzed by quantitative RT-PCR (qRT-PCR). *PHI-1*, *FLG22-INDUCED RECEPTOR-LIKE KINASE 1 (FRK1)*, *CYTOCHROME P450 (CYP81F2)* and *RETICULINE-OXIDASE HOMOLOGUE* (*RET-OX*) genes, here indicated as early elicitor-induced genes, because their expression reaches a maximum within 1 hour (Denoux *et al.,* 2008; Savatin *et al.,* 2014a; Gravino *et al.,* 2015), were analyzed in a time course up to 3 hours. In addition, the expression of late genes that, after elicitor treatment, reach maximal induction at about 3 hours [*POLYGALACTURONASE-INHIBITING PROTEIN 1* (*PGIP1*) and *PHYTOALEXIN DEFICIENT 3* (*PAD3*)] or at least 8 hours [*PLANT DEFENSIN 1.2* (*PDF1.2*) and *PR1*], here indicated as late 1 (L1) and late 2 (L2) genes, respectively, was analyzed. In both AvrPto lines, among the early genes, expression of *FRK1*, *CYP81F2* and *RET-OX* but not that of *PHI-1* was reduced in response to OGs, compared to the wild type, at all time points analyzed (Fig. 3a). On the other hand, the expression of all four genes was severely affected in response to flg22 (Fig. S2a), as expected (He *et al.,* 2006). Reduction of *FRK1*, *CYP81F2* and *RET-OX* transcripts in AvrPto #5.1 seedlings was more moderate than in #4.1 (Fig. 3a and S2a).

The L1 genes *PAD3* and *PGIP1* showed a basal expression 2-3 fold higher than that of the wild type and accumulated at a significantly lower level in response to both OGs and flg22 in both AvrPto lines (Fig. 4a). The L2 genes *PDF1.2* and *PR1* showed an increased basal expression (more than 150-fold and 7-fold higher, respectively) respect to the wild type, higher (for *PDF1.2*) or comparable (*PR-1*) than the expression normally induced by OGs and flg22 in the wild type. Both genes were not further up-regulated by either elicitors (Fig. 4a).

AvrPto affects basal resistance to *B. cinerea* and protection induced by both OGs and flg22

The effect of AvrPto on the OG-induced protection against pathogens was examined. Leaves of β-estradiol-pretreated plants were drop-inoculated with *B. cinerea* conidia 24 hours after water, OG or flg22 spray-pretreatment; disease lesions were measured 48 hours post inoculation (hpi). In the absence of pretreatment with OGs or flg22, AvrPto #4.1 plants showed disease lesions that were statistically larger (40%) than in wild-type plants; moreover, no protection was observed after the treatment with both elicitors (Fig. 5a). The AvrPto 5.1 line displayed no protection against *B. cinerea* after OGs or flg22 pre-treatment, as observed in line 4.1; however basal resistance to the fungus was less affected than in AvrPto #4.1 plants (Fig. 5a), indicating that the presence of AvrPto affects basal resistance to *B. cinerea* and abolishes protection induced by both OGs and flg22.

AvrPto does not affect OG-induced inhibition of auxin-related gene expression

Both OGs and MAMPs regulate also developmental responses, likely due to their ability to antagonize auxin through mechanisms that are still unknown (Savatin *et al.,* 2011). Whether the auxin antagonistic activity of OGs and MAMPs is affected in AvrPto seedlings was investigated by analyzing the expression of the auxin-regulated genes *INDOL-3-ACETIC ACID-INDUCED PROTEIN5* (*IAA5*), *SMALL AUXIN UP RNA* (*SAUR*) *AC1* (*SAUR-AC1*) and *SAUR16*, upon 1 h treatment with IAA, IAA+OGs or IAA+flg22. Expression of the three genes induced by IAA was comparable in the AvrPto lines and similar to that of the wild type, although basal expression in transgenic lines was lower. Upon IAA+OG co-treatment both AvrPto lines showed a normal inhibition of IAA-induced expression of all three genes (Fig. S3); whereas, inhibition did not take place upon flg22+IAA co-treatment, in agreement with the notion that all flg22 responses are inhibited by AvrPto (Fig. S3).

Loss of BAK1 and BKK1 affects only a subset of the OG-induced defense responses that are affected by AvrPto

The contribution of BAK1 and BKK1 to the OG-induced responses described above was further analyzed using the *bak1-5* and *bkk1-1* single mutants (see molecular characterization of the mutants in Fig. S1c) and the *bak1-5 bkk1-1* double mutant. Both single mutants showed no significant alteration of the OG-induced responses, and only *bak1-5* showed defects in the response to flg22, namely MAPKs activation (Fig. S4), the oxidative burst (Fig. S5a), up-regulation of all defense response marker genes (Fig. S6), in agreement with literature data (Roux *et al.,* 2011; Schwessinger *et al.,* 2011); flg22-induced callose deposition and protection against *B. cinerea* were also defective in this mutant (Fig. S5b and S7).

Defective responses were instead observed in the *bak1-5 bkk1-1* double mutant treated with OGs, but only in a subset of the readouts that emerged as affected by the expression of AvrPto (Table 1). Like in AvrPto lines, H_2O_2 accumulation, callose deposition, the induced-protection (Fig. 2a and b and 5b) as well as OG-induced expression of *FRK1, PDF1.2* and *PR1* (Fig. 3b and 4b) were all impaired compared to the wild type. Basal expression of *PDF1.2* was also significantly higher, although not as high as that normally observed upon elicitation with OGs in wild-type seedlings or in AvrPto lines (Fig. 4b). On the other hand, unlike in AvrPto plants, the OG-induced expression of *CYP81F2*, *RET-OX*, *PAD3* and *PGIP1* was not affected, nor their basal expression (Fig. 3b and 4b). MAPK phosphorylation and inhibition of auxin response induced by OGs were normal (Fig. 1 and S3).

All responses to flg22 were strongly reduced (Fig. 1, 2a and b, 4b, 5b and S2b), with the unexpected exception of the inhibition of the auxin-induced gene expression (Fig. S3).

OGs strongly up-regulate *PROPEP2* and *PROPEP3* and loss of PEPR1 and PEPR2 affects the OG-induced expression of *PR1* and protection against *B. cinerea*

Microarray data indicate that OGs, like MAMPs, strongly activate the expression of *PROPEP2* and *PROPEP3* (Denoux *et al.,* 2008). We confirmed these results by analyzing, through qRT-PCR, the induction of *PROPEP2* and *PROPEP3* in response to OGs and, in parallel, to flg22 and elf18. Expression of *PROPEP2* was induced as early as 30 min in wild-type seedlings by all three elicitors and in a similar manner, whereas that of *PROPEP3* was more highly induced by flg22 and elf18 compared to OGs (Fig. 6a). Ethylene signaling has been shown to mediate elf18-triggered expression of *PROPEP2*, but not that of *PROPEP3* (Tintor *et al.,* 2013). The induction of the two genes in response to OGs was therefore analyzed in the *ein2-5* mutant, which is strongly impaired in ethylene signaling (Alonso *et al.,* 1999). Since this mutant shows a low responsiveness to flg22 due to a lower expression of *FLS2* (Mersmann *et al.,* 2010; Boutrot *et al.,* 2010), but normally senses elf18 and OGs (Tintor *et al.,* 2013; Gravino *et al.,* 2015), only the latter MAMP was used in our analysis. At 30 min, expression of *PROPEP2* induced by both OGs and elf18 was reduced, whereas that of *PROPEP3* was even enhanced, compared to the wild type (Fig. 6b). The OG- as well as flg22- and elf18-mediated induction of these two genes was also evaluated in the *cpk5 cpk6 cpk11* triple mutant (Boudsocq *et al.,* 2010), which is defective in OG-triggered ethylene production (Gravino *et al.,* 2015). Induction of both genes by all three elicitors was significantly reduced in the *cpk5 cpk6 cpk11* mutant (Fig. 6a). These data suggest that the CPK5, CPK6 and CPK11 are required for the elicitor-induced expression of both *PROPEP2* and *PROPEP3*, whereas EIN2 is required only for the induction of *PROPEP2*.

The possible role of Peps in OG signaling was investigated, by using a *pepr1 pepr2* double mutant. Among the responses analyzed in the AvrPto plants and in the *bak1 bkk1* mutant, only OG-induced expression of *PR1* and protection against *B. cinerea* were affected in the

pepr1 pepr2 double mutant. Both responses were defective also in response to flg22, along with the expression of *PDF1.2* (Fig. 7a and b). Moreover, our experiments confirm that *PEPR1* and *PEPR2* are involved in the basal resistance against *B. cinerea* (Liu *et al.,* 2013), since lesions caused by this fungus were significantly larger in water-sprayed leaves of the mutant compared to wild type (Fig. 7b). Responses to OGs and flg22 that were not affected in the *pepr1 pepr2* mutant are shown in Fig. S4, S5a and b, S8a and b. All the results obtained with the AvrPto plants, and the *bak1 bkk1* and *pepr1 pepr2* mutants are summarized in Table 1.

DISCUSSION

This work deepens our understanding of the OG signaling, by uncovering that AvrPto affects OG-triggered immunity, beside MAMP-triggered immunity, and demonstrating an important role of the pattern-recognition co-receptors BAK1 and BKK1 as well as of the receptors PEPR1 and PEPR2. Our results also show that perception and early transduction of the OG signal are much more complex than those of flg22. Whereas all the flg22-induced defense responses analyzed here, with one exception that is discussed below, are affected by AvrPto and the lack of BAK1 and BKK1, only a subset of those induced by OGs is affected by AvrPto, and among these, only a subset is affected by the lack of BAK1 and BKK1 (see Table 1).

OG-induced ROS production, callose deposition, up-regulation of *FRK1*, *PDF1.2* and *PR1* as well as protection against *B. cinerea* are all affected by both AvrPto and the lack of BAK1/BKK1, demonstrating that both co-receptors play an important role in OG signaling. These responses are not affected in the corresponding single mutants, indicating a redundant and equal contribution of these LRR-RLKs.

Notably, AvrPto affects OG-induced responses that are BAK1/BKK1-independent, i.e. the up-regulation of the expression of the pairs *CYP81F2/RET-OX* and *PAD3/PGIP1* (partially

and completely, respectively, inhibited in the high-expressing AvrPto line), as well as the activation of MPK3 and MPK6. The latter effect was not observed in the low-expressing AvrPto line, which nevertheless shows partial inhibition of the expression of all four genes, indicating that inhibition of OG-induced MPK3 and MPK6 phosphorylation requires levels of AvrPto higher than those affecting expression of these genes. Our observations provide further evidence that a defective elicitor-induced defense response gene expression may occur in the presence of an apparently unaffected phosphorylation of MPK3 and MPK6 (Schwessinger *et al.,* 2011; Savatin *et al.,* 2014a). Thus, MPK3 and MPK6 phosphorylation may be not sufficient for full activation of the immune gene expression response by elicitors.

Conversely, the induction of the expression of *PHI-1* and the antagonism with auxin emerged as responses totally independent from AvrPto, BAK1 and BKK1. An intriguing result is the independence from BAK1/BKK1 of the flg22-induced inhibition of auxin-regulated gene expression, a response related to the growth-defense trade off that is instead suppressed by AvrPto. At present this is the only flg22 response with this feature.

Both the presence of AvrPto and the lack of BAK1 and BKK1 lead to a higher susceptibility to *B. cinerea,* in agreement with previous results (Zhang *et al.,* 2013) and to higher basal expression of *PDF1.2* (much more conspicuous in AvrPto plants). The latter observation suggests that, in normal conditions, this gene is under a negative action of BAK1 and BKK1 as well as of other elements that are also targets of AvrPto, and that overexpression of *PDF1.2* alone is not sufficient for resistance against *B. cinerea*.

The existence of subsets of OG responses that are differentially affected in the mutants analyzed strongly suggests that different types of perception/transduction complexes mediate OG signaling. Responses that are both AvrPto- and BAK1/BKK1-dependent point to the existence of perception/transduction complex(es) that function mainly through BAK1 and BKK1. AvrPto-dependent BAK1/BKK1-independent responses, instead, point to

different types of complex(es), which may comprise co-receptors other than BAK1 and BKK1 and/or receptors of a different class, targeted however by AvrPto. The behavior of the low-expressing AvrPto line, which shows inhibition of the OG-induced up-regulation of *RET-OX*, *CYP81F2*, *PAD3* and *PGIP1*, albeit at a partial extent, but not of MPK3 and MPK6 activation, may reflect a further heterogeneity in the OG-triggered BAK1/BKK1-independent perception/transduction pathways and therefore different genetic requirement. For example, up-regulation of *PGIP1* and *PAD3* appears to be downstream of receptor/transduction complex(es) that can be completely blocked by AvrPto, whereas that of *CYP81F2* and *RET-OX* may involve also elements with low or no affinity for AvrPto. Similarly, phosphorylation of MAPKs may be mediated by receptor/co-receptor kinases or other types of kinases also characterized by a low affinity for AvrPto.

Finally, a third type of complexes, not requiring the function of BAK1 and BKK1 and capable of eluding the suppression action of the effector, may be involved in the OG responses that are independent of AvrPto, BAK1 and BKK1. The possibility exists that a combinatorial interaction between different receptors and intracellular, extracellular or plasma membrane co-receptors leads to the observed complexity. Moreover, each type of receptor complex may be redundant.

Members of the SERK family other than BAK1 and BKK1, i.e. *SERK1* and *SERK2* [*SERK5* is described to be defective in the Col-0 ecotype used in this work (Wu *et al.,* 2015)], are good candidates for playing a role in OG- and flg22-triggered BAK1/BKK1-independent responses. The role of these proteins in OG-induced responses has never been investigated. As FLS2 is considered the only receptor of flg22, and SERK1 and SERK2 have been demonstrated to be recruited to the FLS2 perception complex (Roux *et al.,* 2011), inhibition by flg22 of auxin-regulated gene expression, an AvrPto-dependent response, may be mediated specifically by these co-receptors. Our results are consistent with the hypotheses that direct targets of AvrPto are the co-receptors SERKs, or FLS2

alone, as previously proposed (Xiang *et al.,* 2008). On the other hand, antagonism with auxin is independent not only from BAK1/BKK1 but also from AvrPto in the case of OGs, suggesting that it may be downstream of receptor/co-receptors unique to the OG perception/signaling pathway.

Whether WAK1, the only OG receptor identified so far, interacts with AvrPto or BAK1, BKK1 or other SERKs is not yet known and, in general, none of the five WAK family members has been so far identified among the interactors of either AvrPto or BAK1 (Bogdanove and Martin, 2000; Halter *et al.,* 2014). AvrPto has been shown to interact with receptor kinases of different classes, e.g. the LRR-type receptors FLS2, EFR, and BAK1, and the LysM-type CERK1, besides interacting directly with the protein kinase Pto in tomato cells (Zipfel and Rathjen, 2008; Gimenez-Ibanez *et al.,* 2009); thus, an interaction of AVrPto with WAKs cannot be ruled out, considering the difficulties in the extraction and purification of these proteins (He *et al.,* 1996; Wagner and Kohorn, 2001; Decreux and Messiaen, 2005).

Among the danger signal so far characterized, the complexity of the OG signaling is unprecedented. Such a complexity has long been envisioned, since no mutants have been so far identified that are completely insensitive to these elicitors. Moreover, a redundant role of the different WAK members in the OG perception is likely, but not yet demonstrated, although WAK2, besides WAK1, can bind *in vitro* OGs and pectin (Decreux and Messiaen, 2005; Decreux *et al.,* 2006; Kohorn *et al.,* 2012).

A multiplicity of perception/transduction complexes for OGs, as suggested by this work, may recall the complexity of perception/early transduction of the signaling system that can be considered the vertebrate analogue of that represented by homogalacturonan/OGs in plants (Cervone *et al.,* 2015). This involves the extracellular matrix component hyaluronan (HA) and its fragments, which are produced after tissue injury or upon enzymatic activity of hyaluronidases, either synthesized by animal cells or secreted by pathogens (Kreil, 1995),

and induce different inflammatory-related responses in different cell-types and tissues, so that HA is now thought as an immune regulator in human diseases (Jiang *et al.,* 2011). Both HA and HA fragments are bound by different and numerous proteins, both membrane-linked and extracellular, such as the PRRs Toll-like receptors TLR2 and TLR4, CD44, a polymorphic type I transmembrane glycoprotein known as the major cell-surface HA binding protein, RHAMM (receptor for hyaluronan-mediated motility expressed protein) and BREVICAN (Jiang *et al.,* 2011; Lee-Sayer *et al.,* 2015). In this case, signaling results from the specific combination of different elements depending on tissue- and cell-type and from the situation that needs to be perceived, e.g. infectious *versus* noninfectious tissue injury. Signaling mediated by animal PRRs, including the different TLRs, is highly complex (Tan *et al.,* 2014) and in most cases involves combinatorial and/or sequential stimulation of different perception/transduction complexes, allowing to activate different immune responses in a synergistic manner, thus amplifying the signal, and/or in a compensatory manner, providing robustness to the signaling system. The combined action of different elements allows also a fine tuning of the immune response, in order to discriminate between pathogenic or nonpathogenic microbes, and in general to efficiently respond to different types of pathogens (Kim *et al.,* 2014). A similar complexity may exist in signaling mediated by homogalacturonan-OGs.

Finally, we show here that also *PEPR1* and *PEPR2* are shared elements between OG and MAMP signaling and are required for *PR1* up-regulation and protection against *B. cinerea* induced by OGs or flg22. Both responses are dependent on the ethylene signaling pathway (Tintor *et al.,* 2013; Gravino *et al.,* 2015). Because *PEPR1* and *PEPR2* are required for a proper response to ethylene, as 1-aminocyclopropane-1-carboxylic acid (ACC)-induced seedling growth inhibition, gene expression and protection against *B. cinerea* are affected in the *pepr1 pepr2* mutant (Liu *et al.,* 2013), the requirement of these receptors is likely due to their role in ethylene response. Moreover, OG- and elf18-induced

up-regulation of *PROPEP2*, but not of *PROPEP3,* is Et-dependent. Thus, as in the case of elf18 (Tintor *et al.,* 2013), Pep2 is likely to be a player in the cascade that links OG perception to ethylene and the downstream responses, i.e. *PR1* induction and acquired resistance against *B. cinerea* (Gravino *et al.,* 2015). Because *PROPEP1* is induced only at a limited extent (about 2-fold) by OGs, whereas *PROPEP2* and *PROPEP3* are strongly induced (Denoux *et al.,* 2008), Pep1 is unlikely to play a major role in OG-induced protection against *B. cinerea*, although this peptide has been shown to enhance resistance to this fungus (Liu *et al.,* 2013).

PEPR1 and *PEPR2* are instead dispensable for MAPK activation and ROS production by both OGs and flg22 [this work, (Krol *et al.,* 2010; Bartels *et al.,* 2013)], and for all the other responses induced by these elicitors (Table 1), except for the up-regulation of *PDF1.2*, which occurs at a similar extent in response to the two elicitors in the wild type but appears to require *PEPR1* and *PEPR2* only in the case of flg22. This defect is unlikely to be due to the reduced responsiveness to ethylene of the *pepr1 pepr2* mutant (Liu *et al.,* 2013), since OG-induced *PDF1.2* expression is also strongly dependent on the ethylene signaling pathway (Gravino *et al.,* 2015). The basis of this different behavior therefore is not obvious, unless OGs activate an additional ethylene-dependent but PEPR1/PEPR2-indepent pathway that leads to the up-regulation of this gene. This pathway may be activated by at least one of different perception/transduction complexes that we propose here to mediate response to OGs. Our identification of the distinct subsets of OG reads out provides specific markers that may help to elucidate the complexity of the OG signaling.

EXPERIMENTAL PROCEDURES

Plant growth and treatment

Arabidopsis (*Arabidopsis thaliana*) Columbia-0 (Col-0) wild-type seeds were purchased from Lehle Seeds. The *bkk1-1* (salk_057955), *pepr1-1* (salk_059281) and *pepr2-1* (salk_098161) single mutants (in Col-0 background) were purchased from The European Arabidopsis Stock Centre. The *bak1-5* and *bak1-5 bkk1-1* mutants (in Col-0 background) were kindly provided by Cyril Zipfel (The Sainsbury Laboratory, Norwich Research Park, Norwich, NR4 7UH, United Kingdom). The *pepr1-1 pepr2-1* double mutant was generated by crossing the *pepr* single mutants. Conditional AvrPto expressing plants were generated by expressing AvrPto coding sequence in Col-0 under an estradiol-inducible promoter. The DNA sequence encoding AvrPto was amplified from pET29a plasmid kindly provided by Jen Sheen (Department of Molecular Biology, Massachusetts General Hospital, Department of Genetics, Harvard Medical School, Boston, USA). The cassette containing Avrpto and the beta-estradiol inducible system was obtained by using the Gateway Recombination Cloning Technology (Life Technologies) and the gateway-compatible pMDC7 binary vector (Karimi *et al.,* 2002), obtained from Plant System Biology (Ghent University; http:// gateway.psb.ugent.be/). T1 independent plants were screened by antibiotic (hygromycin) resistance and analyzed by PCR analysis (see primer sequences in Table S1) for the presence of AvrPto transcripts in excised leaves treated with beta-estradiol (1 µM) for 48 hours. Among the homozygous T3 plants two lines (#4.1 and #5.1) were chosen for the analyses.

For treatments of seedlings, seeds were surface sterilized and grown in multi-well plates (approximately 10 seeds/well), containing 2 mL per well of Murashige and Skoog (MS) medium [SIGMA ALDRICH; (Murashige and Skoog*,* 1962)] supplemented with 0.5% sucrose, at 22°C and 70% relative humidity under a 16/8 hour light/dark cycle

(approximately 120 µmol m^{-2} s^{-1}). AvrPto transgenic seedlings were grown in the presence of 1 µM β-estradiol or DMSO, as a control, from 48 h before analyses. After 9 days, the medium was adjusted to a final volume of 1 mL and treatments with water, OGs (40 or 50 µg mL^{-1}), flg22 (10 nM) or elf18 (10 nM) were performed after an additional day. Analysis of the auxin antagonistic action of OGs or flg22 were performed as previously described (Savatin *et al.,* 2011; Savatin *et al.,* 2014a). Root length analysis was performed in Col-0 and AvrPto transgenic seedlings grown for 10 days in MS agar (0.8%) medium supplemented with 1% sucrose and 1 µM β-estradiol or DMSO.

For *B. cinerea* protection assay, 4-week-old plants were sprayed with water, OGs (200 µg mL^{-1}) or flg22 (1 µM). For ROS and callose analyses, 4-week-old plants were treated with OGs (200 µg mL^{-1}) or flg22 (100 nM). AvrPto transgenic plants were sprayed with 10 µM β-estradiol or DMSO, three times within a week, before elicitors treatment. Plants were grown at 22°C and 70% relative humidity under a 12/12 hour light/dark cycle (approximately 120 µmol m^{-2} s^{-1}).

OGs with an average DP of 10 to 16, as assessed by matrix-assisted laser desorption/ionization time-of-flight MS, were prepared as previously described (Bellincampi *et al.,* 2000). The flg22 and elf18 peptides were synthesized by EZBiolab.

Gene expression analysis

Gene expression analyses were performed as previously described (Savatin *et al.,* 2014a). Primer sequences are shown in Supplemental Table S1.

Immunoblot assay

Immunoblot assays were performed as previously described (Savatin *et al.,* 2014a).

***Botrytis cinerea* protection assay**

Protection assays against *B. cinerea* were performed as previously described (Savatin *et al.,* 2014a).

Measurement of ROS

ROS measurements were performed as previously described (Gigli *et al.,* 2015).

Callose deposition

Callose deposition was performed as previously described (Galletti *et al.,* 2008). Callose quantification was performed by using ImageJ software.

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Supporting Information

Supporting Figure S1. Characterization of mutants and AvrPto-expressing plants.

Supporting Figure S2. Flg22-triggered induction of early defense response genes is affected in AvrPto-expressing and bak1-5 bkk1-1 mutant seedlings.

Supporting Figure S3. Inhibition of auxin-regulated gene expression by OGs is not affected in AvrPto- expressing and bak1-5 bkk1-1 mutant seedlings.

Supporting Figure S4. MAPK activation induced by OGs is not affected in *bak1-5*, *bkk1-1* and *pepr1 pepr2* mutants.

Supporting Figure S5. ROS production and callose deposition in response to OGs are not impaired in the single mutants *bak1-5* and *bkk1-1* and in the double mutant *pepr1 pepr2*.

Supporting Figure S6. OG-triggered induction of early and late defense response genes is not affected in the *bak1-5* and *bkk1-1* single mutants.

Supporting Figure S7. OG-induced protection against *B. cinerea* is not affected in the *bak1-5* and *bkk1-1* single mutants.

Supporting Figure S8. OG-triggered induction of early and late defense response genes and inhibition of auxin-regulated gene expression are not affected in the *pepr1 pepr2* double mutant.

Supporting Table S1. Primers used in this work.

Table 1: Behavior[a] of the immune responses induced by OGs and flg22 in the mutant/transgenic plants.

Response	Timing[b]	OG			flg22		
		AvrPto	*bak1-5 bkk1*	*pepr1 pepr2*	AvrPto	*bak1-5 bkk1*	*pepr1 pepr2*
Protection to *B. cinerea*	late 2	↓	↓	↓	↓	↓	↓
PR1 up-regulation	late 2	↓	↓	↓	↓	↓	↓
PDF1.2 up-regulation	late 2	↓	↓	=	↓	↓	↓
Callose deposition	late 2	↓	↓	=	↓	↓	=
ROS production	early	↓	↓	=	↓	↓	=
FRK1 up-regulation	early	↓	↓	=	↓	↓	=
CYP81F2 up-regulation	early	↓	=	=	↓	↓	=
RET-OX up-regulation	early	↓	=	=	↓	↓	=
PAD3 up-regulation	late 1	↓	=	=	↓	↓	=
PGIP1 up-regulation	late 1	↓	=	=	↓	↓	=
MAPK activation	early	↓	=	=	↓	↓	=
PHI-1 up-regulation	early	=	=	=	↓	↓	=
Inhibition of auxin responses	early	=	=	=	↓	=	=

[a] ↓: affected (reduced); =: not affected.
[b] Phase in which each response reaches maximal expression. Early: maximal response up to around 1 h, late 1 and late 2: maximal response around 3 h and at least 8 h, respectively.

Table S1. Primers used in this work.

GENE	AGI CODE	FORWARD PRIMER (5'-3')	REVERSE PRIMER (5'-3')
UBQ5	AT3G62250	GTTAAGCTCGCTGTTCTTCAGT	TCAAGCTTCAACTCCTTCTTTC
BAK1[*]	AT4G33430	GGCTTGCGTATTTACATGATCATTA	GGTGGGTTGGGTAGTTGAAA
RET-OX	AT1G26380	AGGTTCTCGAACCCTAACAACA	GCACAGACGACACGTAAGAAAG
CYP81F2	AT5G57220	GTGAAAGCACTAGGCGAAGC	ATCCGTTCCAGCTAGCATCA
FRK1	AT2G19190	TTAAACTCGACGATGCAACA	GATGGAAGTTTTCCCGTTTT
PAD3	AT3G26830	CCGGTGAATCTTGAGAGAGCC	GATCAGCTCGGTCATTCCCC
PHI-1	AT1G35140	TTGGTTTAGACGGGATGGTG	ACTCCAGTACAAGCCGATCC

PR1	AT2G14610	GTAGGTGCTCTTGTTCTTCCC	CACATAATTCCCACGAGGATC
PDF1.2	AT5G44420	CGCACCGGCAATGGTGG	ATCCATGTTTGGCTCCTTCG
PGIP1	AT5G06860	TCTTGAACTTAGCAGGAAC	GAGAGCTGGTTATGTGATAG
IAA5	AT1G15580	ACCGAACTACGGCTAGGTCT	CTGTTCTTTCTCCGGTACGA
SAUR16	AT4G38860	ACAATGCTACGACGAGGAAG	CTTCTTCACAAGGGATGGTG
SAUR-AC1	AT4G38850	AAGGGAATCATCGTCGACAC	TATTGTTAAGCCGCCCATTG
WRKY33	AT2G38470	GAAACAAATGGTGGGAATGG	TGTCGTGTGATGCTCTCTCC
AvrPto		GGGGACAAGTTTGTACAAAAAAG CAGGCTCCATGGGAAATATATGT GTCGGC	GGGGACCACTTTGTACAAGAAAG CTGGGTAAGCGTAGTCTGGAACG TCGT

Figure legends:

Figure 1. MAPK activation induced by OGs is partially affected only in AvrPto-expressing line 4.1, but not in line 5.1 and *bak1-5 bkk1-1* double mutant. Levels of phosphorylated MPK3 and MPK6 (pMPK3 and pMPK6) in seedlings of wild type (Col-0), AvrPto-expressing lines (#4.1 and #5.1) and *bak1-5 bkk1-1* after elicitation with OGs (40 µg mL^{-1}) or flg22 at the indicated time points were determined by immunoblot analysis using an anti-p44/42-ERK antibody (top panels). Levels of MPK3 and MPK6 total proteins were determined using specific antibodies (bottom panels). The identity of individual MAP kinases, as determined by size, is indicated by arrows. Experiments were repeated three times with similar results.

Figure 2. ROS production and callose deposition in response to OGs are impaired in AvrPto- expressing and *bak1-5 bkk1-1* double mutant plants. (a) ROS production, expressed in relative light units (RLUs), was measured in leaf discs of wild type (Col-0), AvrPto expressing lines (#4.1 and #5.1) and *bak1-5 bkk1-1* mutant plants during elicitation with water, OGs or flg22. Results are average ± SE (n = 12). (b) Callose deposition

visualized by aniline blue staining in leaves of Col-0, AvrPto expressing lines (#4.1 and #5.1) and *bak1-5 bkk1-1* mutant plants 24 h after syringe-infiltration with water, OGs or flg22. All images are at the same scale (scale bar = 0.2 mm; 10× magnification). The average number of callose deposits (± SE) of ten different leaf samples for each treatment is indicated in the images. Experiments in (a) and (b) were repeated three times with similar results.

Figure 3. OG-triggered induction of only a subset of early defense response genes is affected in AvrPto-expressing and *bak1-5 bkk1-1* mutant seedlings. Expression of the indicated early-induced defense marker genes was analyzed by qRT-PCR in seedlings of wild type (Col-0) and AvrPto-expressing lines (#4.1 and #5.1) (a) or *bak1-5 bkk1-1* mutant (b) after treatment with OGs (40 µg mL^{-1}) at the indicated time points. Transcripts levels are shown as the mean of at least three independent experiments (± SE; n = 20 in each experiment) normalized to *UBQ5* expression and plotted relative to expression in water-treated Col-0. In (a) differences in OG-triggered induction of *RET-OX*, *FRK1* and *CYP81F2,* at 30, 60 and 180 minutes, between Col-0 and both 4.1 and 5.1 lines, were statistically significant (Student's *t* test; $P < 0.01$); in (b) asterisks indicate statistically significant differences between transgenic/mutant and wild-type samples at the indicated time points, according to Student's *t* test (*, $P < 0.05$; **, $P < 0.01$).

Figure 4. Loss of BAK1 and BKK1 affects only a subset of the OG-induced late defense response genes that are affected by AvrPto. Expression of the indicated late-induced defense marker genes, analyzed by qRT-PCR in seedlings of the wild type (Col-0) and AvrPto-expressing lines (#4.1 and #5.1) (a) or *bak1-5 bkk1-1* mutant (b) after 3 h (for *PAD3* and *PGIP1*) and 8 h (for *PDF1.2* and *PR1*) of treatment with water, OGs (50 µg mL^{-1}) and flg22. Transcript levels are shown as the mean of at least three independent experiments (± SE; n = 20 in each experiment) normalized to *UBQ5* expression and

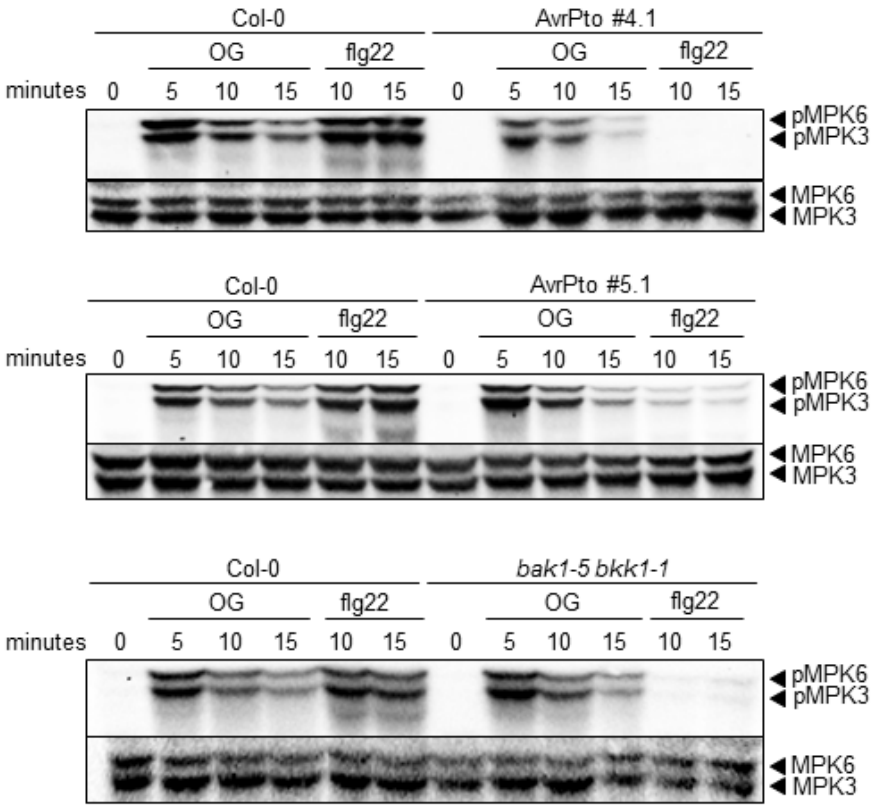
plotted relative to expression in water-treated Col-0. In (a) and (b) asterisks indicate statistically significant differences between mutant and wild-type samples, according to Student's *t* test (*, P < 0.05; **, P < 0.01).

Figure 5. Basal resistance and elicitor-induced protection against *B. cinerea* are affected in AvrPto- expressing and *bak1-5 bkk1-1* mutant plants. Wild-type (Col-0) plants and AvrPto-expressing lines (#4.1 and #5.1) (a) or *bak1-5 bkk1-1* mutant plants (b) were sprayed with water, OGs or flg22 and, after 24 hours, leaves were inoculated with *B. cinerea* spores. Lesion areas were measured at 48 hpi. Results are average ± SE (n = 20 lesions). Different letters above bars indicate statistically significant differences between samples, as determined by ANOVA with Tukey-HSD test [P < 0.05 in (a); P < 0.01 in (b)]. The experiments were repeated three times with similar results.

Figure 6. OG-triggered induction of the expression of *PROPEP2*, but not of *PROPEP3*, is dependent on ethylene. Expression of *PROPEP2* and *PROPEP3,* analyzed by qRT-PCR in seedlings of wild type (Col-0) and *cpk5 cpk6 cpk11* mutant (a) or *ein2-5* mutant (b) after 30 min of treatment with water, OGs (50 µg mL^{-1}), flg22 and elf18 as indicated. Transcript levels are shown as the mean of at least three independent experiments (± SE; n = 20 in each experiment) normalized to *UBQ5* expression and plotted relative to expression in water-treated Col-0. In (a) and (b) asterisks indicate statistically significant differences between mutant and wild-type samples, according to Student's *t* test (*, P < 0.05; **, P < 0.01).

Figure 7. OG- and flg22-triggered induction of *PR1* expression and protection against *B. cinerea* are affected in the *pepr1 pepr2* double mutant. (a) Expression of *PR1* and *PDF1.2,* analyzed by qRT-PCR in seedlings of the wild type (Col-0) and the

pepr1 pepr2 mutant 8 h after treatment with water, OGs (50 µg mL^{-1}) and flg22. Transcript levels are shown as the mean of at least three independent experiments (± SE; n = 20 in each experiment) normalized to *UBQ5* expression and plotted relative to expression in water-treated Col-0. Asterisks indicate statistically significant differences between mutant and wild-type samples, according to Student's *t* test (*, $P < 0.05$; **, $P < 0.01$). (b) Col-0 and *pepr1 pepr2* mutant plants were sprayed with water, OGs or flg22 and, after 24 h, leaves were inoculated with *B. cinerea* spores. Lesion areas were measured at 48 hpi. Results are average ± SE (n = 20 lesions). Different letters above bars indicate statistically significant differences between samples, as determined by ANOVA with Tukey-HSD test ($P < 0.05$). The experiment was repeated three times with similar results.



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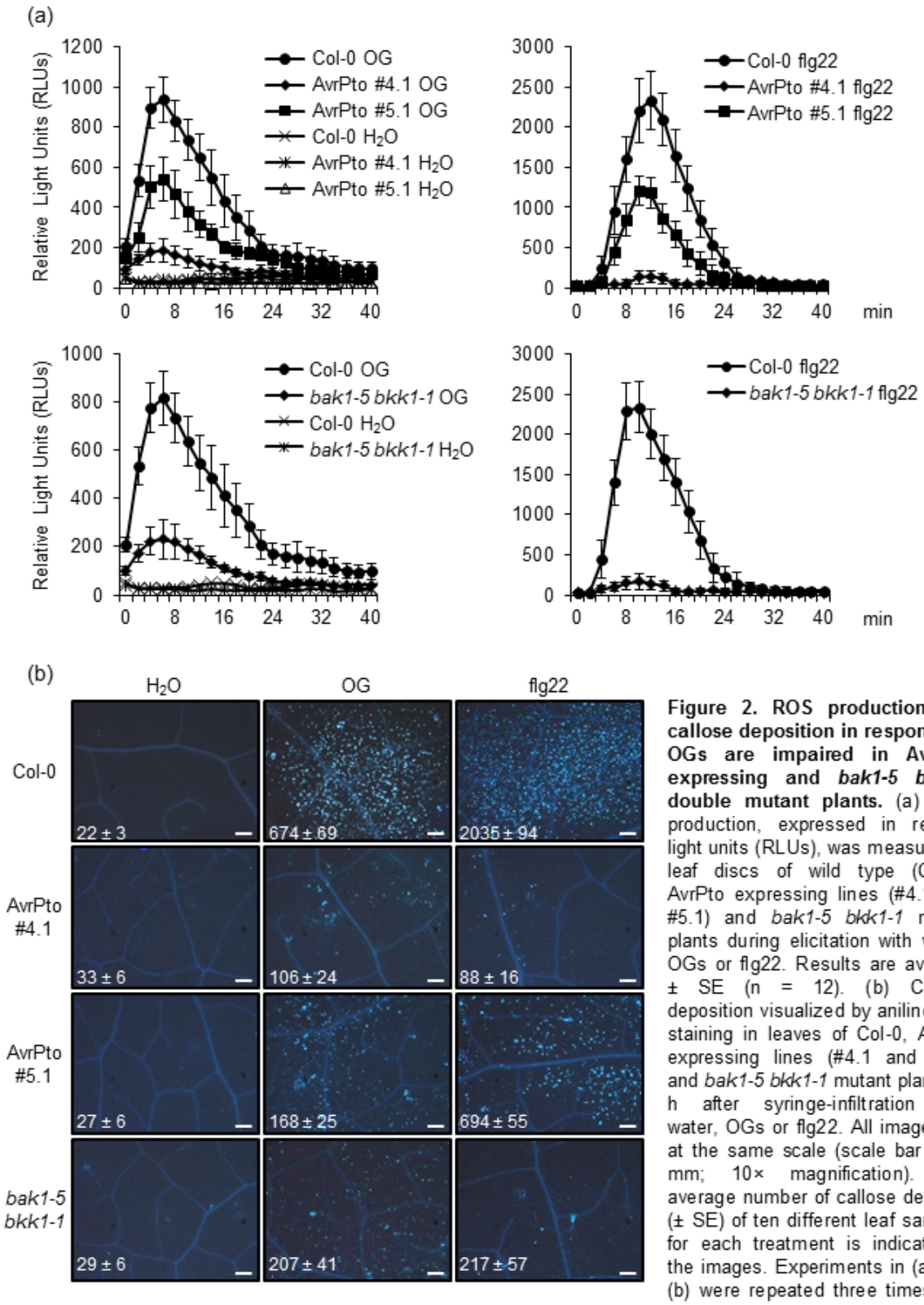

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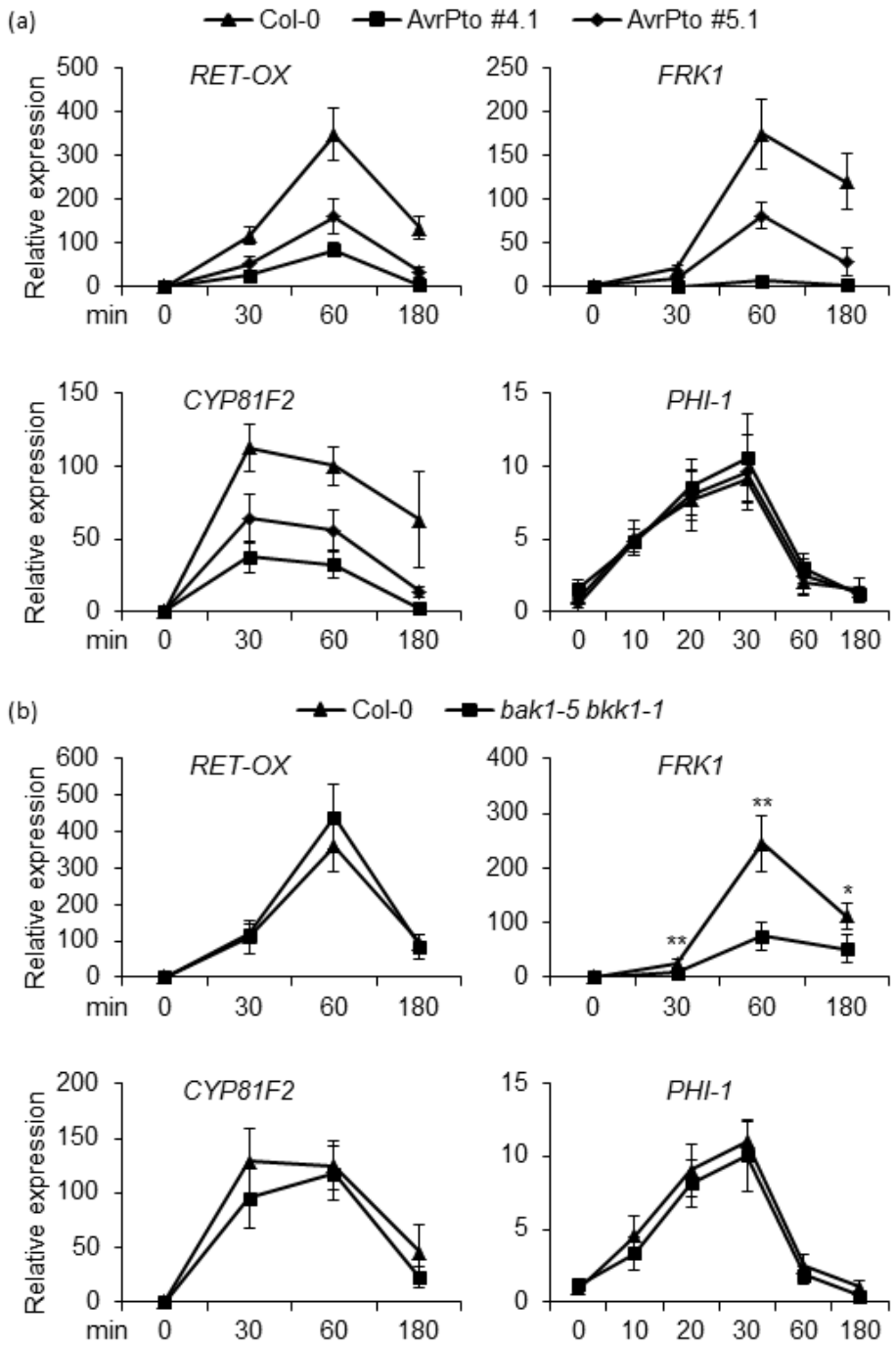

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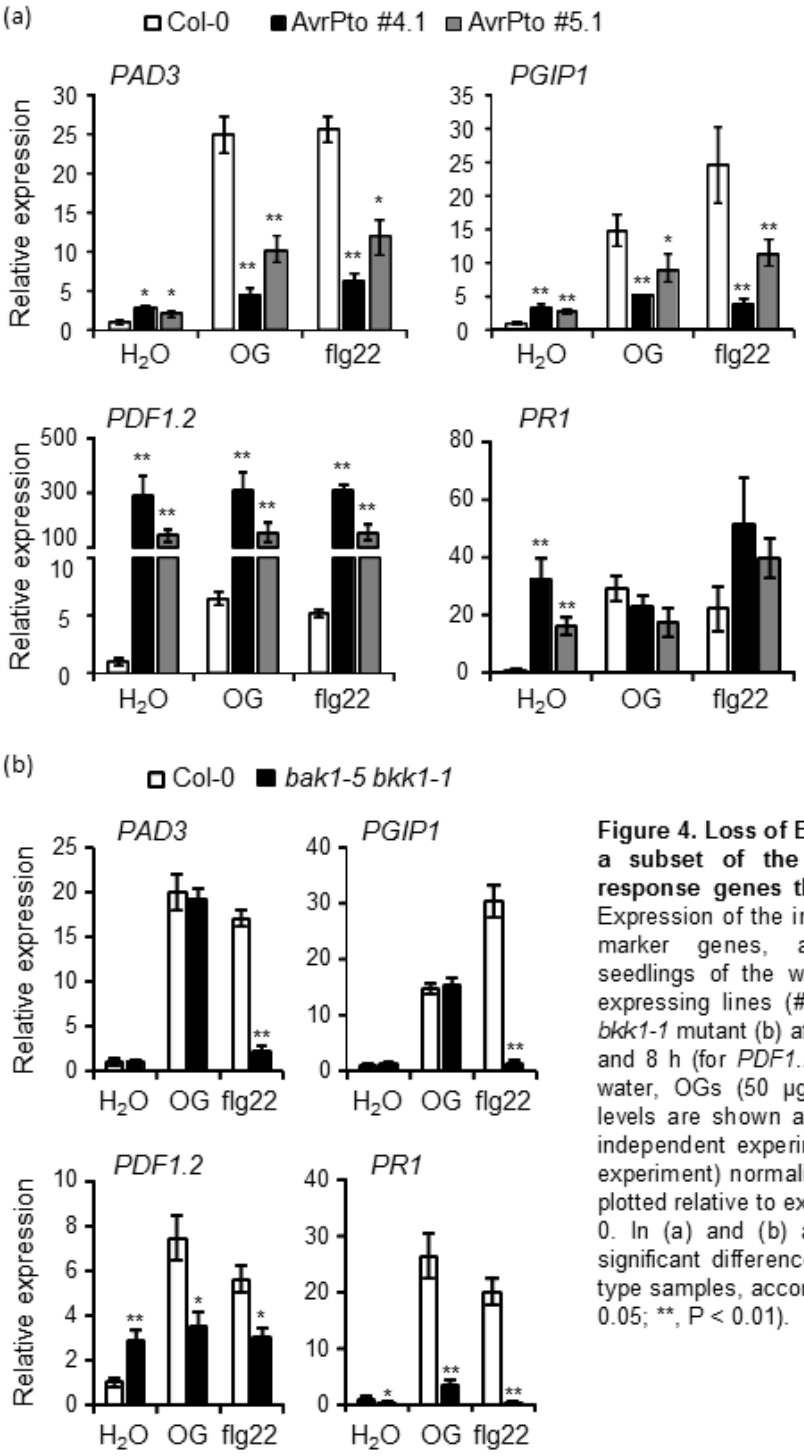

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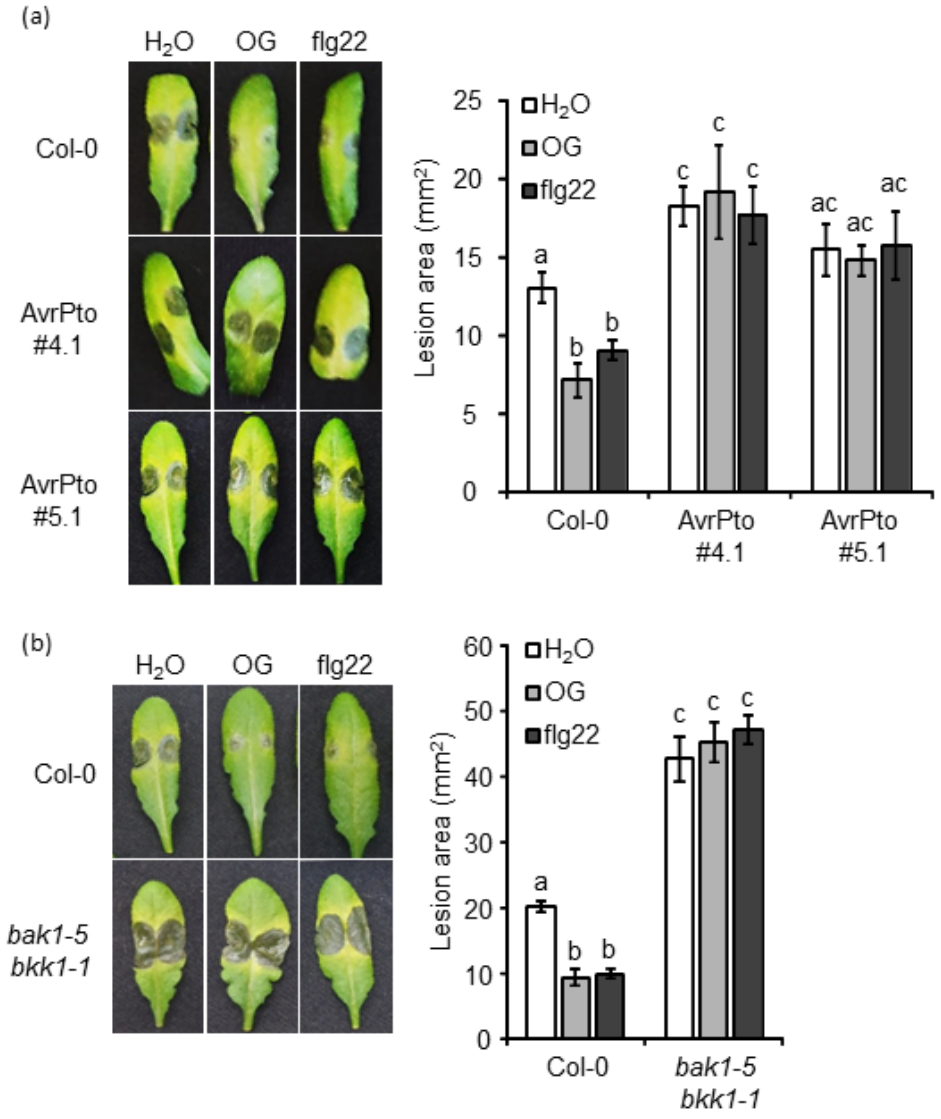

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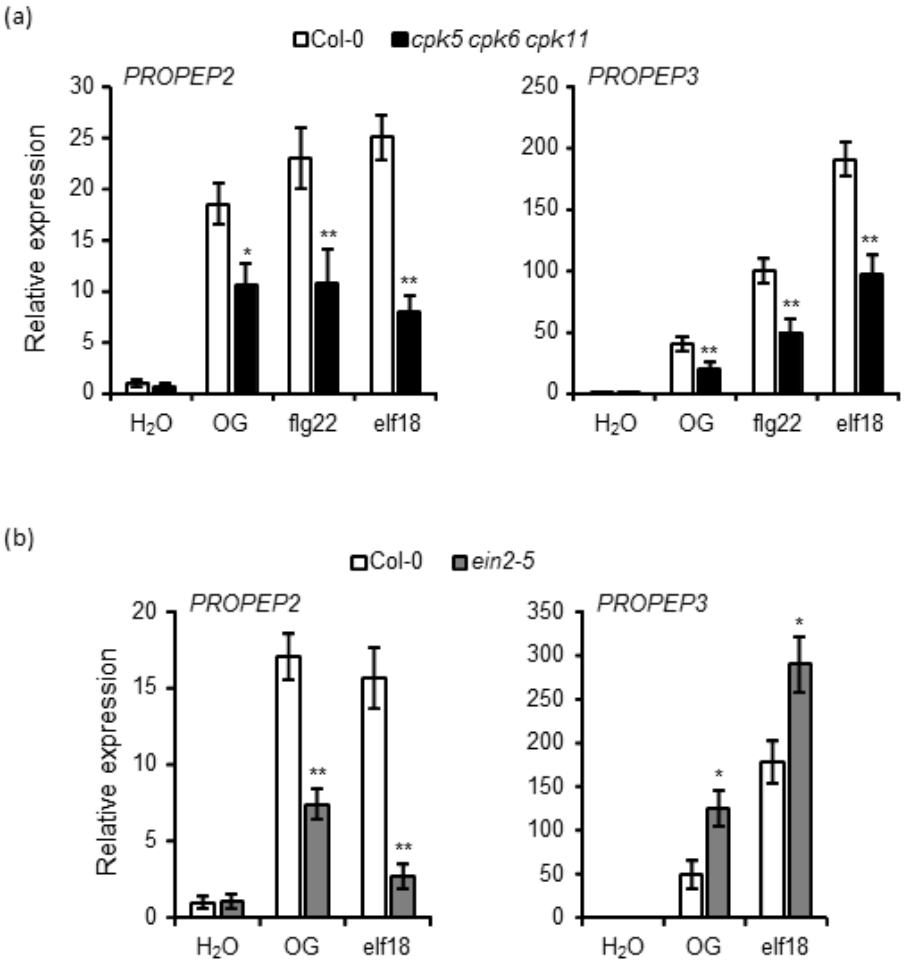

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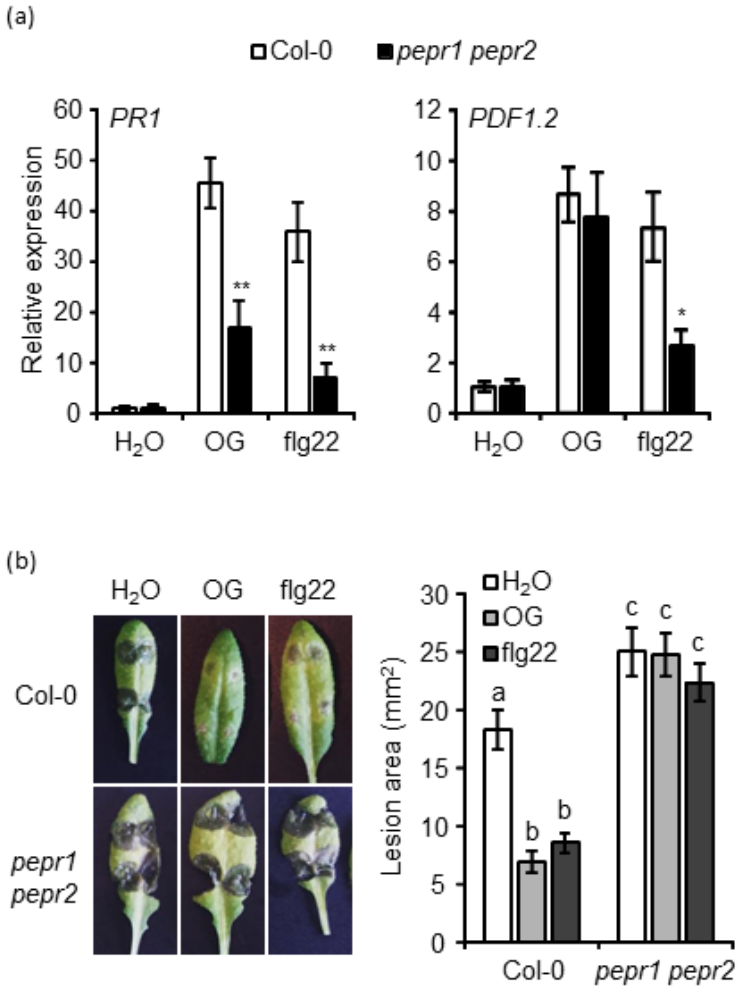

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