

A protein phosphatase 2C, responsive to the bacterial effector AvrRpm1 but not to the AvrB effector, regulates defense responses in Arabidopsis

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SUMMARY

Using a proteomics approach, a PP2C-type phosphatase (renamed PIA1, for PP2C induced by AvrRpm1) was identified that accumulates following infection by *Pseudomonas syringae* expressing the type III effector AvrRpm1, and subsequent activation of the corresponding plant NB-LRR disease resistance protein RPM1. No accumulation of PIA1 protein was seen following infection with *P. syringae* expressing AvrB, another type III effector that also activates RPM1, although PIA transcripts were observed. Accordingly, mutation of PIA1 resulted in enhanced RPM1 function in response to *P. syringae* pathover tomato (*Pto*) DC3000 (*avrRpm1*) but not to *Pto* DC3000 (*avrB*). Thus, PIA1 is a protein marker that distinguishes AvrRpm1- and AvrB-dependent activation of RPM1. AvrRpm1-induced expression of the pathogenesis-related genes *PR1*, *PR2* and *PR3*, and salicylic acid accumulation were reduced in two *pia1* mutants. By contrast, expression of other defense-related genes, including *PR5* and *PDF1.2* (plant defensin), was elevated in unchallenged *pia1* mutants. Hence, PIA1 is required for AvrRpm1-induced responses, and confers dual (both positive and negative) regulation of defense gene expression.

Keywords: PP2C, pathogenesis-related, salicylic acid, *Pseudomonas syringae*, disease resistance.

INTRODUCTION

The first step in immunity is the recognition of pathogen-associated molecular patterns (PAMPs), which results in PAMP-triggered immunity (PTI) (Boller and Felix, 2009). During evolution, pathogens have acquired PTI-interfering effector proteins to render certain plant species susceptible. In turn, plants evolve disease resistance proteins that recognize the presence of individual effectors, resulting in effector-triggered immunity (ETI) (Chisholm *et al.*, 2006; Grant *et al.*, 2006; Jones and Dangl, 2006). In contrast to PTI, ETI is usually specific to one effector, and conforms to the 'gene-for-gene' interaction model postulated by Flor (1971). Natural selection subsequently drives pathogens to avoid ETI by diversifying the recognized effectors or by acquiring new effectors to suppress ETI and permit infection. Plants may then acquire new resistance proteins to counteract new

effectors. This alternation in resistance status constitutes the 'zigzag' model proposed by Jones and Dangl (2006) to describe the plant-pathogen 'arms race' that propels the survival of either the plant or the pathogen.

The 'gene-for-gene' model is characterized by genetic interaction between pathogen *avr* (avirulence) gene loci and alleles of the corresponding plant disease resistance (*R*) locus (Van der Biezen and Jones, 1998; Dangl and Jones, 2001). This is often manifested in programmed cell death (the hypersensitive response, HR) to halt pathogen growth, and is thus a very effective defense response (Hammond-Kosack and Jones, 1996; McDowell and Woffenden, 2003). Direct R-Avr interactions have been demonstrated for the flax L resistance protein and AvrL of flax rust fungus (Dodds *et al.*, 2006), rice Pi-ta and Avr-Pita of *Magnaporthe grisea*

(Jia *et al.*, 2000), and Arabidopsis RSS1-R and Pop2 of *Ralstonia solanacearum* (Deslandes *et al.*, 2003). For many other R–Avr pairs, no physical interaction has been shown. As an alternative, the ‘guard hypothesis’, in which plant R proteins recognize Avr proteins through their action on host targets, has been proposed (Van der Biezen and Jones, 1998). RPM1-interacting protein 4 (RIN4) is an example of a host target protein whose the modification by Avr proteins is ‘guarded’ by R proteins. Two effectors of *Pseudomonas syringae*, AvrRpm1 and AvrB, can stimulate phosphorylation of RIN4, which is thought to then be perceived by RPM1, the cognate R protein for AvrRpm1 and AvrB. Another *P. syringae* effector, AvrRpt2, also targets RIN4, causes its disappearance through protease activity, and activates the defense response conferred by RPS2 (Mackey *et al.*, 2002, 2003). In the absence of RPM1 and RPS2, AvrRpm1 and AvrRpt2 manipulate RIN4 in order to suppress PTI and promote virulence of the corresponding *P. syringae* strains in these plants (Belkhadir *et al.*, 2004; Kim *et al.*, 2005a,b). However, it is not known how such target modifications benefit pathogen virulence.

Despite substantial progress in understanding NB-LRR-mediated disease resistance, downstream defense signaling pathways have not been defined. A frequent event in signal transduction pathways is reversible protein phosphorylation, which provides a mechanism to regulate protein conformation, activity, localization and stability (Hunter, 1995). In addition to kinases, protein phosphatases are emerging as regulators of defense responses in plants. AP2C1, an Arabidopsis PP2C phosphatase, negatively regulates the mitogen-activated protein kinases MPK4 and MPK6, as well as resistance to necrotrophic pathogens, whereas WIN2, a PP2C that interacts with the bacterial HopW1-1 effector, is required for resistance against virulent *P. syringae* (Schweighofer *et al.*, 2007; Lee *et al.*, 2008). In the AvrRpm1–RPM1 system, phosphorylation of signaling components, such as RIN4, has been shown, but the kinases and phosphatases involved are not known.

In a proteomics-based study, a PP2C accumulating during the AvrRpm1–RPM1 interaction was identified (Widjaja *et al.*, 2009). We show here that this specific PP2C accumulation is induced by AvrRpm1, but not AvrB. Loss of this PP2C increased RPM1 function, affected the expression of subsets of *PR* (pathogenesis-related) and stress-related genes, and resulted in lower salicylic acid (SA) accumulation after bacterial infection.

RESULTS

To identify potential components of the AvrRpm1–RPM1 signaling pathway, proteins that are differentially regulated during this interaction were analyzed by two-dimensional electrophoresis in transgenic Arabidopsis lines conditionally expressing *avrRpm1* (Widjaja *et al.*, 2009). MALDI-TOF mass spectrometry identified one of these up-regulated proteins,

with sequence coverage of approximately 62% (Figure S1), as a PP2C encoded by At2g20630 (which we renamed as PIA1, PP2C induced by AvrRpm1). The AvrRpm1-induced PIA1 accumulation is rather low – it is not detectable in total or microsomal protein extracts prior to fractionation steps that deplete abundant proteins (e.g. Rubisco) and its visualization required silver staining (Widjaja *et al.*, 2009). The rapid accumulation of this phosphatase (i.e. 1 h after *avrRpm1* expression is detectable) suggests potential role(s) for PIA1 in early RPM1-mediated signaling.

PIA1 up-regulation is specific for the AvrRpm1–RPM1 interaction

Kim *et al.* (2009) showed that residual defense activation occurs in *rpm1-3* plants through weak ‘off target’ activation of another NB-LRR protein, RPS2, by AvrRpm1. However, PIA1 up-regulation is strictly RPM1-dependent as it is only observed in *RPM1*, and not in *rpm1-3*, lines (Figure 1a,b).

PIA1 accumulation observed in the transgenic DEX-inducible *avrRpm1* system (Figure 1) can be reiterated by infiltration of wild-type *Arabidopsis thaliana* (Col-0) with *Pto* DC3000 (*avrRpm1*), which expresses and naturally delivers AvrRpm1 into plant cells during infection (Figure 2a). To analyze whether PIA1 accumulation is induced by other bacterial effectors, Arabidopsis Col-0 plants were infiltrated with *Pto* DC3000 (*avrB*), which also activates RPM1-mediated disease resistance (Mackey *et al.*, 2002), or *Pto* DC3000 (*avrRpt2*), which activates RPS2-mediated

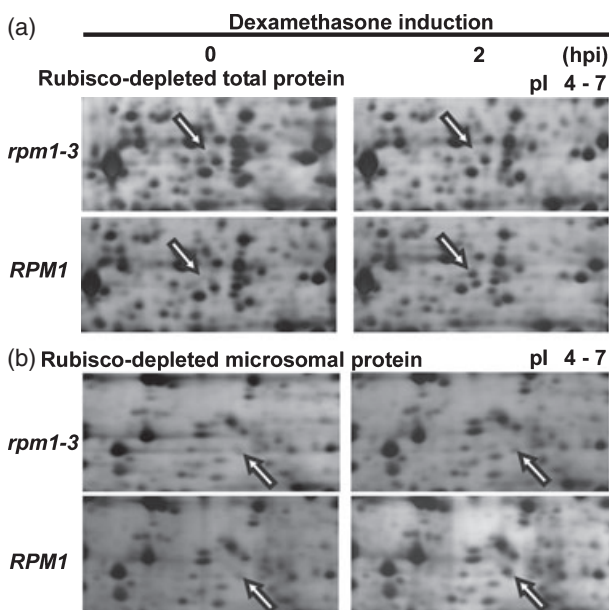


Figure 1. PP2C up-regulation after dexamethasone treatment in the *RPM1* line, but not the *rpm1-3* line.

Panels of selected regions of two-dimensional electrophoresis gels showing the PP2C spot (indicated by arrows) before and after dexamethasone treatment (2 hpi) in Rubisco-depleted total protein (a) and Rubisco-depleted microsomal protein (b) fractions. The peptide mass-fingerprinting-based identification of the PP2C spot (renamed PIA1) is shown in Figure S1.

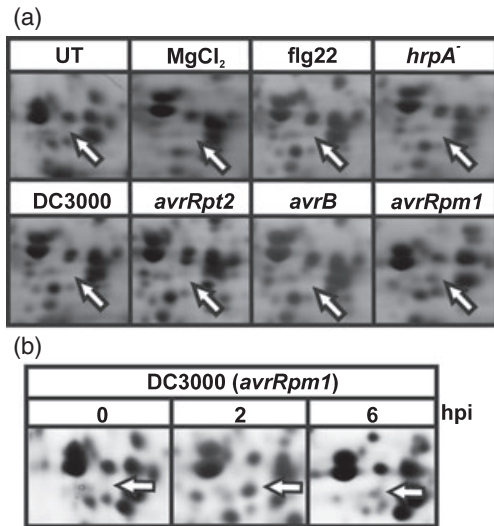


Figure 2. Only *Pto* DC3000 (*avrRpm1*) infiltration led to PIA1 accumulation. (a) Selected regions from two-dimensional electrophoresis gels showing PIA1 (indicated by arrows) before (UT, untreated) and 2 h after infiltration with MgCl₂, flg22 (10 μM), a type III secretion system mutant (*hrpA*), virulent *Pto* strain (DC3000), or various avirulent strains expressing the indicated *avr* genes at 10⁸ cfu ml⁻¹. (b) Time course of PIA1 accumulation after *Pto* DC3000 (*avrRpm1*) infiltration.

disease resistance (Mackey *et al.*, 2003). *Pto* DC3000, *Pto* DC3000 (*hrpA*) and flg22 were included to compare the PIA1 response to the whole arsenal of type III effectors, a type III secretion system mutant or a PAMP, respectively. Two-dimensional electrophoresis analysis showed that, among this set of stimuli, PIA1 was up-regulated exclusively by infiltration of bacteria with *avrRpm1* (Figure 2a). The PIA1 protein spot could be clearly seen 2 h after treatment and at a lower level after 6 h (Figure 2a,b). Surprisingly, no up-regulation of PIA1 was seen following inoculation with *Pto* DC3000 (*avrB*) (Figure 2a), although PIA1 transcripts did accumulate after infiltration with both *avrB*- or *avrRpm1*-expressing strains (see Figure 5). Hence, of the two bacterial effectors that are known to activate RPM1, PIA1 protein accumulation appears to be specific to AvrRpm1, and this is probably regulated at the post-transcriptional level.

At2g20630 T-DNA insertion mutants do not express PIA1

To investigate the role of PIA1 in RPM1-mediated defense responses, two SALK T-DNA insertion lines for At2g20630 were isolated, and the positions of the T-DNA inserts were determined by DNA sequencing. The insert is located in the second exon of At2g20630 in the first line (N519305), and at the border between the third exon and the adjacent intron of At2g20630 in the second line (N605978) (Figure 3a). Neither mutant showed accumulation of either PIA1 mRNA (Figure 3b) or PIA1 protein (Figure 3c) after infiltration with *Pto* DC3000 (*avrRpm1*). Thus, the two *pia1* lines are null

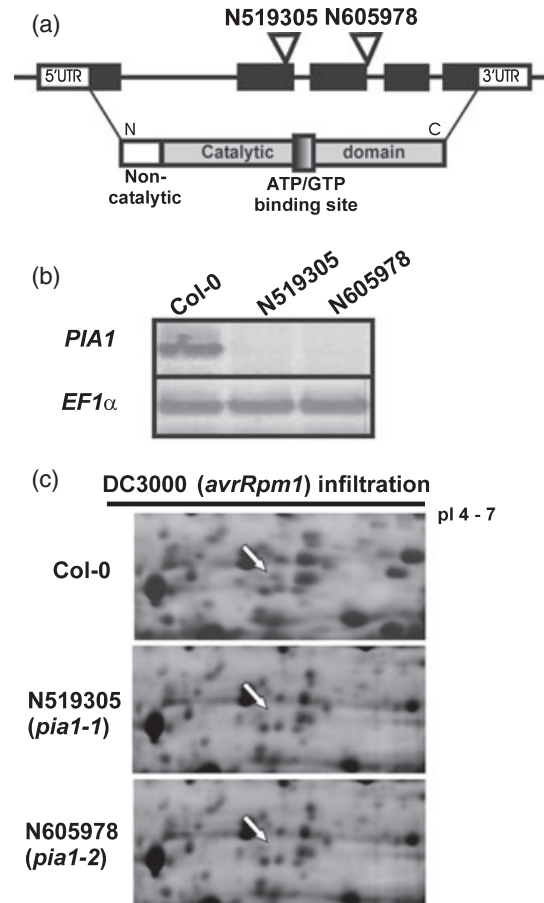


Figure 3. Two *Arabidopsis* PIA1 mutants (N519305 and N605978) do not express PIA1 mRNA and protein (after *Pto* DC3000 (*avrRpm1*) infiltration, 2 hpi). (a) Schematic diagram showing the PIA1 gene (exon-intron) structure and PIA1 protein motifs. The positions of the T-DNA insertion are indicated by triangles. (b) PIA1 mRNAs were absent in the two mutants (as determined by RT-PCR). (c) No PIA1 protein accumulated at the expected position (as determined by two-dimensional electrophoresis) in the two mutants compared to Col-0.

mutants, and are designated hereafter as *pia1-1* and *pia1-2*, respectively (Figure 3c).

The *pia1* mutants show enhanced disease resistance against *Pto* DC3000 (*avrRpm1*), but not against *Pto* DC3000 (*avrB*) or *Pto* DC3000

The *pia1* mutants do not exhibit any obvious phenotype under normal growth conditions. To check whether PIA1 alters other defense responses, such as the HR or ROS accumulation, Col-0 and the *pia1* mutants were infiltrated with *Pto* DC3000 (*avrRpm1*) and, after 7 h, subjected to trypan blue (Peterhänzel *et al.*, 1997) and 3,3-diaminobenzidine (DAB) (Thordal-Christensen *et al.*, 1997) staining to indicate cell death and H₂O₂ accumulation, respectively. No distinction could be made between Col-0 and the *pia1* lines, suggesting that neither the HR nor ROS accumulation are considerably affected by

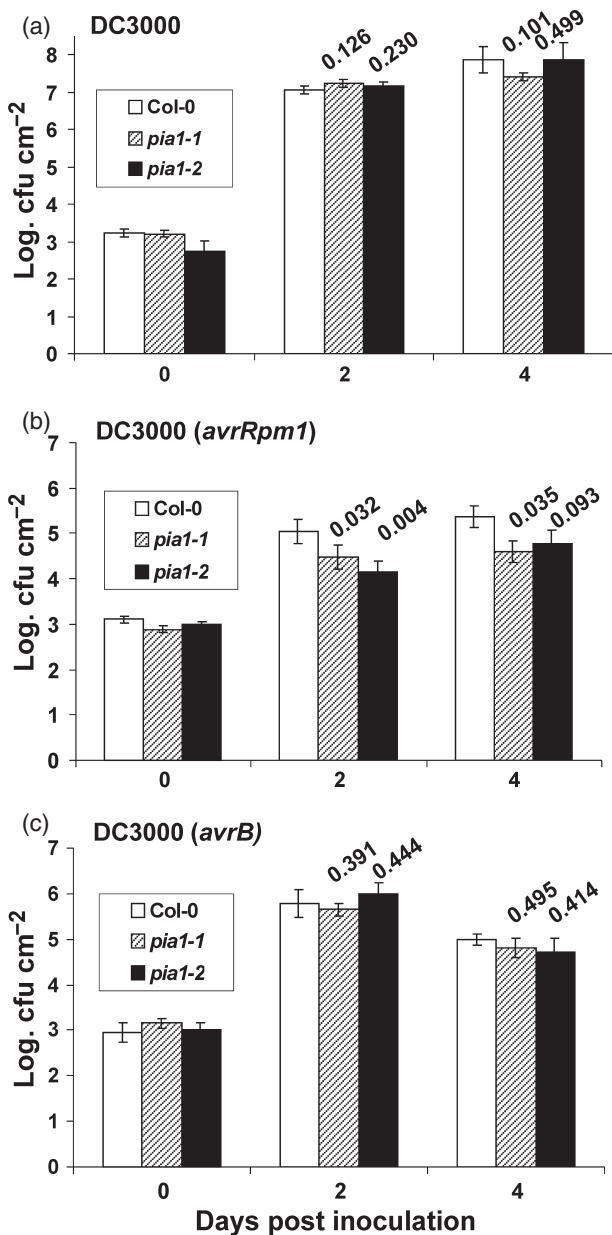


Figure 4. Loss of *PIA1* did not change the susceptibility to the virulent *Pto* DC3000 strain or avirulent strain *Pto* DC3000 (*avrB*) (a,c), but increased resistance to the avirulent strain, *Pto* DC3000 (*avrRpm1*) (b). Plants were syringe-infiltrated with bacteria resuspended at 10^5 cfu ml⁻¹, and bacterial growth was monitored. Data are means \pm SE for 18 plants (DC3000) or 28 plants (DC3000 (*avrRpm1*)). The *P* values for the *t* test analysis, compared to the corresponding Col-0 plants, are indicated above each column. The experiment was repeated four times for *Pto* DC3000, twice for *Pto* DC3000 (*avrB*) and five times for *Pto* DC3000 (*avrRpm1*), with similar results.

the loss of *PIA1* (Figure S2). Lower-dose bacterial inoculation to evoke weaker reactions for detecting subtle phenotypes did not result in any obvious difference (data not shown).

As impairment of pathogen colonization is not always achieved through development of an HR (Hammond-Kosack and Jones, 1996), bacterial growth was analyzed. Growth of

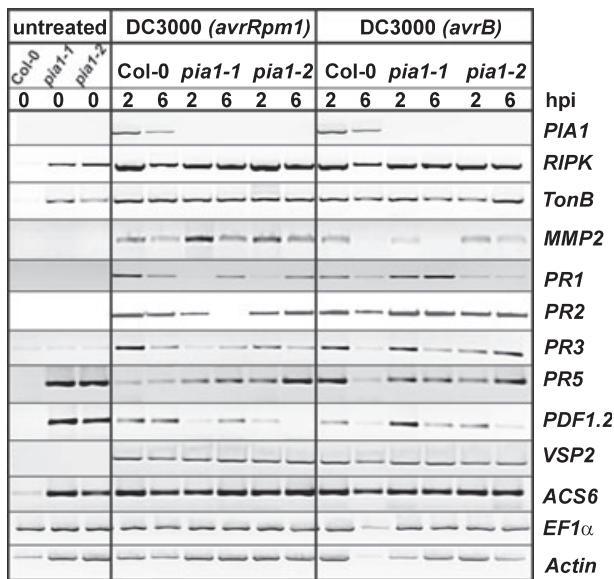


Figure 5. Loss of *PIA1* changed the expression pattern of several defense- and stress-related marker genes before and after infiltration with *Pto* DC3000 (*avrRpm1*) or *Pto* DC3000 (*avrB*).

The transcript levels were monitored by semi-quantitative RT-PCR, with EF1 α and actin as constitutive controls. The experiment was repeated twice with similar results. Abbreviations: RIPK, RPM1-induced protein kinase; TonB, putative chloroplast protein; MMP2, matrix metalloprotease; ACS6, ACC synthase; PR, pathogenesis-related; PDF1.2, plant defensin; VSP2, vegetative storage protein.

the virulent strain *Pto* DC3000 and the avirulent strain *Pto* DC3000 (*avrB*) did not differ between Col-0 and the *pia1* mutants (Figure 4a,c). By contrast, growth of *Pto* DC3000 (*avrRpm1*), which normally proliferates poorly in Col-0 due to activation of RPM1-dependent disease resistance, was reduced approximately fivefold in the *pia1* mutants compared to Col-0 at 2 and 4 days after inoculation (Figure 4b). Although the difference in bacterial growth is modest, it is reproducible (in five repetitions), and indicates that loss of *PIA1* enhanced the response to avirulent *Pto* DC3000 (*avrRpm1*) but not avirulent *Pto* DC3000 (*avrB*).

PIA1 regulates the expression of several RPM1-responsive, pathogenesis- and stress-related genes

The observation that the loss of *PIA1* did not affect HR formation, but further increased resistance against avirulent *Pto* DC3000 (*avrRpm1*), suggests that *PIA1* affects *AvrRpm1*-dependent activation of RPM1 specifically. To test this hypothesis, expression of three marker genes for RPM1-mediated responses [*RPM1*-induced protein kinase (*RIPK*; At2g05940), a matrix metalloprotease (*MMP2*; At1g70170) and a gene encoding a protein of unknown function but predicted to localize to the chloroplast (*TonB*; At2g32190)] (de Torres *et al.*, 2003) was analyzed by semi-quantitative RT-PCR. After *Pto* DC3000 (*avrRpm1*) treatment, there were

enhanced *MMP2* transcript levels in the *pia1* mutants compared to Col-0, but *RIPK* and *TonB* were not affected (Figure 5). By contrast, expression of the *PR* genes *PR1/2/3* and of plant defensin (*PDF1.2*) was slightly reduced or delayed in the *pia1* mutants compared to Col-0 (Figure 5).

Many of the *PR* genes tested above are controlled by SA (Dong, 1998). To evaluate the contribution of other major defense-related hormonal pathways, we checked expression of marker genes for jasmonic acid (JA) signaling (*VSP2*) and ethylene (ET) biosynthesis. The basal levels of *ACS6* (1-aminocyclopropane-1-carboxylic acid (ACC) synthase-6) were higher in the *pia1* mutants, but the pathogen-induced expression of *VSP2* and *ACS6* in the *pia1* mutants was similar to Col-0 (Figure 5).

When compared to housekeeping genes such as *EF1 α* , in addition to *ACS6* already mentioned above, higher expression of *PR5*, *PDF1.2* and, to a lesser extent *RIPK* and *TonB*, was seen in the *pia1* mutants prior to infection (Figure 5, left panel). Together, these results suggest that PIA1 is required to suppress the expression of subsets of genes (*PR5*, *PDF1.2*, *ACS6*, *RIPK* and *TonB*) prior to infection, and it is required for full expression of *PR1*, *PR2* and *PR3* during the *RPM1*-mediated response.

In agreement with the apparent lack of a role for PIA1 in defense against *Pto* DC3000 (*avrB*) (Figure 4c), expression patterns after infiltration with *Pto* DC3000 (*avrB*) were generally similar between Col-0 and *pia1* mutants (Figure 5, right panel). Thus, PIA1 is required for some of the *AvrRpm1*-induced, but not *AvrB*-induced, *RPM1* responses.

Analysis of defense-related phytohormones in *pia1*

SA, JA and ET levels were determined to evaluate their possible role in signaling in *pia1* mutants. Although JA levels were slightly lower in one of the *pia1* mutants compared to Col-0 at 6 hours post inoculation (hpi), there was no significant difference in levels of its precursor, 12-oxo-phytodienoic acid (OPDA), between the lines before or after infiltration with *Pto* DC3000 (*avrRpm1*) (Figure 6a,b). In particular, no difference in basal JA levels was observed.

ET levels were similar in Col-0 and the *pia1* mutants under untreated conditions, as well as upon infiltration with *Pto* DC3000 (*avrRpm1*) (Figure 7). As the ET levels after $MgCl_2$ infiltration (wounding control) were much lower compared to those after bacterial infiltration, the wounding effect during infiltration is negligible. However, the ET levels in the *pia1* mutants appear to be lower compared to Col-0 after $MgCl_2$ treatment, and this difference was significant for both mutants (Student's *t* test: $P < 0.05$ for line *pia1-1* and $P < 0.01$ for line *pia1-2*). Thus, PIA1 may affect wound-induced ET accumulation but is not essential in controlling ET levels during interaction with *Pto* DC3000 (*avrRpm1*).

The levels of free SA, SA glycoside (SAG) and total SA in the *pia1* mutants were generally lower compared to those in Col-0 (Figure 8a–c), but statistically significant differences

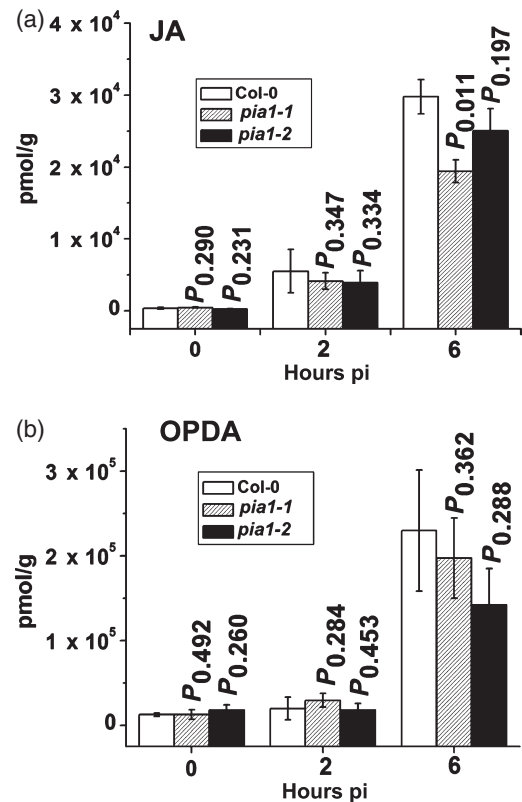


Figure 6. JA (a) and OPDA (b) levels in the *pia1* mutants were generally not different from those in Col-0 after *Pto* DC3000 (*avrRpm1*) infiltration. Although the level of JA was lower at 6 hpi for line *pia1-1*, there was no consistent reduction in either *pia1* mutant. The numbers above the columns are the *t* test *P* values compared to Col-0 as reference. Data are the means of three biological replicates \pm SE.

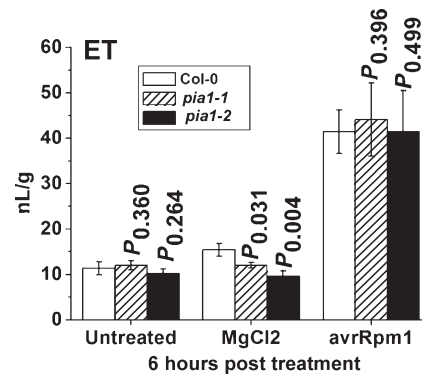


Figure 7. ET levels in the *pia1* mutants and Col-0 were similar at baseline and after *Pto* DC3000 (*avrRpm1*) infiltration, but lower after $MgCl_2$ treatment. The amount of ET accumulating for 6 h after treatment was measured. The values are the means \pm SE of 5–7 plants, and the numbers above the columns are the *t* test *P* values. Four leaves per plant were sampled, and the experiment was repeated twice.

were only found at later time points, such as 6 hpi (Figure 8) or later (data not shown). Thus, PIA1 is required for *AvrRpm1*-induced SA accumulation.

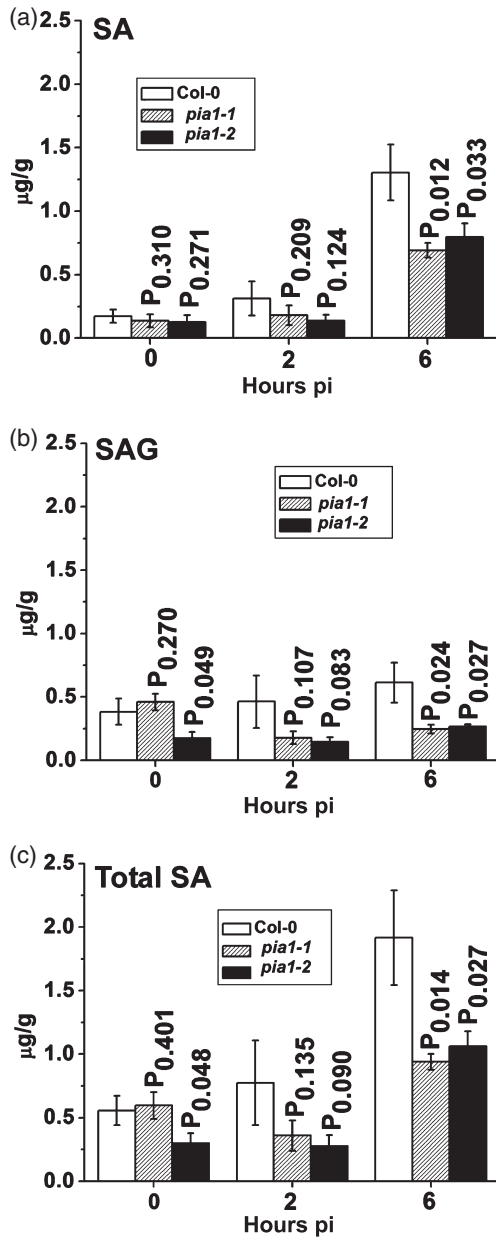


Figure 8. SA, SAG and total SA levels in the *pia1* mutants were significantly reduced compared to *Col-0*.

Plants were infiltrated with *Pto* DC3000 (*avrRpm1*) re-suspended at 10^8 cfu ml^{-1} , and the levels of SA (a), SAG (b) and total SA (c) in infiltrated leaves were measured at 0, 2 and 6 hpi. The data shows the means \pm SE, based on three biological replicates. Numbers above the columns are the *t* test *P* values.

PIA1 does not regulate phosphorylation of RIN4 and AtREM1.2

PIA1 negatively regulates disease resistance against *Pto* DC3000 (*avrRpm1*) (Figure 4), and may be assumed to regulate early phosphorylation events that occur during *AvrRpm1*-induced signaling. Two membrane-associated

proteins are known to be rapidly phosphorylated in response to *AvrRpm1*-dependent activation of RPM1, namely RIN4 (Mackey *et al.*, 2002) and Remorin (AtREM1.2), a putative lipid raft component (Widjaja *et al.*, 2009). Phosphorylated RIN4 can be detected as a transient mobility shift on one-dimensional SDS-PAGE with immunodetection (Mackey *et al.*, 2002) after *Pto* DC3000 (*avrRpm1*) infection (Figure 9a). The phosphorylation pattern was not altered in the *pia1* mutants. Visualization of AtREM1.2 phosphorylation required two-dimensional electrophoresis analysis (Widjaja *et al.*, 2009). AtREM1.2 phosphorylation on two-dimensional electrophoresis was observed in both wild-type and the *pia1* mutants (Figure 9b). Hence, phosphorylation of RIN4 and AtREM1.2 is either not regulated by PIA1, or, alternatively, there are other PP2Cs that can redundantly control the phosphorylation status.

DISCUSSION

The Arabidopsis PP2C family consists of 76 genes that are clustered into ten groups based on amino acid sequence homology (Schweighofer *et al.*, 2004). Biological functions have been assigned only to a few PP2Cs, such as those

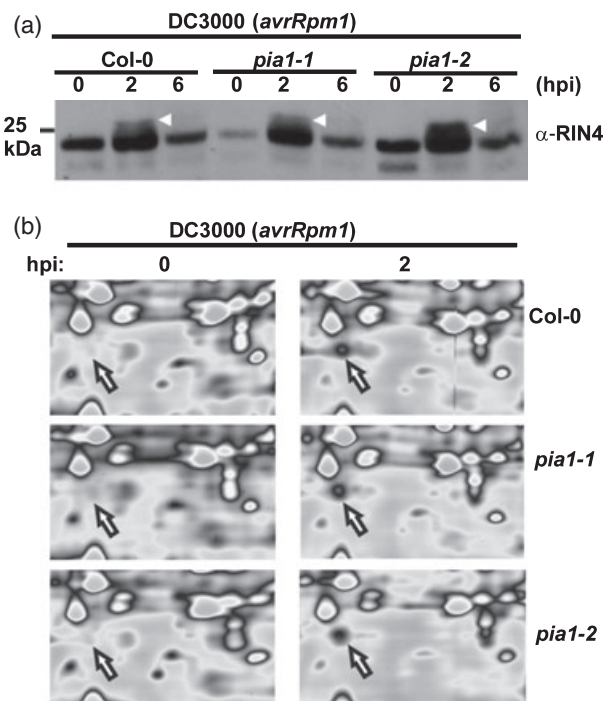


Figure 9. RIN4 and AtREM1.2 phosphorylation was not affected by the loss of PIA1.

Plants were infiltrated with *Pto* DC3000 (*avrRpm1*) re-suspended at 10^8 cfu ml^{-1} .

(a) RIN4 was immunodetected after one-dimensional electrophoretic separation and Western blotting, with phosphorylation detected as a mobility shift (white arrowheads).

(b) AtREM1.2 isoforms were visualized by silver-staining after two-dimensional electrophoresis. Selected regions of the two-dimensional electrophoresis panels are shown, with the position of the major form of phosphorylated AtREM1.2 (showing a shift towards acidic pI) indicated by arrows.

associated with abscisic acid (ABA) perception and signaling (Ma *et al.*, 2009; Park *et al.*, 2009), or the homolog of MP2C, an alfalfa (*Medicago sativa*) PP2C that regulates MAPK signaling (Schweighofer *et al.*, 2004). The PP2C identified in this work, PIA1, is rapidly up-regulated during *AvrRpm1*-dependent RPM1 activation, and has not been described previously. Recently, another member in the same PP2C group, At4g31750, was identified as a HopW1-1-interacting protein (Lee *et al.*, 2008), highlighting the possibility of bacterial effectors targeting certain PP2Cs in order to curb defense responses.

PIA1 negatively regulates disease resistance mediated by RPM1

Reducing phosphatase levels by silencing *PP2Ac* has been shown to increase resistance to virulent *P. syringae* pv. *tabaci* (He *et al.*, 2004), and increasing the levels of AP2C1, an Arabidopsis MAPK-interacting PP2C, compromised immunity against the necrotrophic pathogen, *Botrytis cinerea* (Schweighofer *et al.*, 2007). Resistance to *B. cinerea* was not affected in the *pia1* mutants (data not shown). Thus, PIA1 acts differently from AP2C1-type PP2Cs. Loss of PIA1 led to a small but reproducible enhanced resistance against avirulent *Pto* DC3000 (*avrRpm1*) (Figure 4). This marginal difference is probably due to enhancement of the existing RPM1-mediated responses as no general enhanced resistance phenotype was observed.

The mode of action for the enhanced RPM1 function in *pia1* mutants against *Pto* DC3000 (*avrRpm1*) is currently unknown, but is apparently independent of HR, ROS (Figure S2) or ET/JA accumulation (Figures 6 and 7). The decreased SA accumulation in the *pia1* mutants is not concordant with the enhanced resistance. Additionally, it is also likely that PIA1 acts independently or downstream of proteins that are known to be phosphorylated following delivery of *AvrRpm1* to cells expressing RPM1, such as RIN4 or Remorin (AtREM1.2) (Figure 9).

Of the three genes tested that are considered to be RPM1-regulated markers (de Torres *et al.*, 2003), expression of *MMP2* was slightly stronger in the *pia1* mutants after challenge with *Pto* DC3000 (*avrRpm1*) but not *Pto* DC3000 (*avrB*). This expression pattern correlated with enhanced resistance against *Pto* DC3000 (*avrRpm1*), although it is unclear whether *MMP2* contributes to the *AvrRpm1*-dependent RPM1 response. In this context, a soybean pathogen-responsive matrix metalloprotease, GmMMP2, is thought to digest and release microbial cell wall elicitors that enhance plant defense activation, and recombinant GmMMP2 can inhibit the growth of *P. syringae* pv. *glycinea* and *Phytophthora sojae* (Liu *et al.*, 2001).

PIA1 regulates pathogenesis- and stress-related gene expression

In addition to *MMP2*, other defense genes are also mis-regulated in *pia1* mutants during RPM1-mediated disease

resistance. These can be placed in several categories. In group I, expression of *PR1* and *PR2*, which are SA-inducible (Uknes *et al.*, 1992), is reduced in the *pia1* mutants after infiltration with *Pto* DC3000 (*avrRpm1*), probably corresponding to decreased SA levels in *pia1* mutants. In group II, expression of the JA/ET-regulated *PR3* gene (Van Loon and Van Strien, 1999) is suppressed, like the SA-regulated *PR* genes, but there is no correlation with JA, OPDA or ET levels, which are not altered in the *pia1* mutants. In group III, several genes (*PR5*, *PDF1.2*, *ACS6*, *RIPK* and *TonB*) have elevated basal expression in *pia1* mutants prior to bacterial infection, but this is insufficient to establish an enhanced disease resistance phenotype. Two of these, *PDF1.2* and *PR5*, can be further categorised as a sub-group, where their expression are normally activated after bacterial infection in wild-type plants but are repressed (i.e. lower than in the un-inoculated tissues) after bacterial infection in the mutants. This suggests that PIA1 suppresses their expression under non-challenged conditions, but switches to positive regulation during the *RPM1*-mediated defense response. Taken together, PIA1 appears to have dual roles in controlling subclasses of *PR* gene expression.

In an attempt to determine whether PIA1 controls the expression of these stress-related genes through hormonal control, we measured JA, ET and SA levels. With the exception of group I genes (SA-regulated genes), there was no correlation between *PR* gene expression and hormone levels. For *PDF1.2* expression (group III), both JA and ET signaling are required simultaneously (Penninckx *et al.*, 1998). One may speculate that the constitutive expression of *PDF1.2* in the *pia1* mutants is regulated through ET rather than JA, as the JA-regulated *VSP2* expression in *pia1* is unaffected. However, this is not accompanied by elevated ET levels (Figure 7). Thus, in addition to altering homeostasis of certain phytohormones, PIA1 probably regulates defense gene expression through phosphorylation-related signaling.

Raz and Fluhr (1993) showed that application of phosphatase inhibitors led to expression of *PR1*, *PRB-1b*, *PR2* and *PR3* in tobacco (*Nicotiana tabacum*). By contrast, kinase inhibitors induced *PR1* gene expression, which is suppressed in *NahG* tobacco plants that cannot accumulate SA, thus suggesting a phosphorylation event upstream of SA (Conrath *et al.*, 1997). Although extrapolated from different plant systems, the first finding is in agreement with the current results indicating that expression of some defense genes (group III) is induced when a phosphatase such as PIA1 is missing, while the second finding reflects the PIA1 regulation of SA levels in the *pia1* mutants and the correlation.

PIA1 regulates an *AvrB*-independent pathway in the RPM1-mediated defense response

PIA1 was up-regulated by *AvrRpm1* but not by *AvrB* at the post-transcriptional level (Figures 2 and 5). Both effectors are

often considered to act similarly in RPM1-mediated disease resistance. However, the *P. syringae* pv. *maculicola* strain, *Pma* M6CΔE (*avrRpm1*), but not the corresponding *Pma* M6CΔE (*avrB*) strain, grew 10-fold more than *Pma* M6CΔE (vector) in *rpm1* plants. This suggests an RPM1-independent pathway that is responsive to AvrB but not to AvrRpm1 (Belkhadir *et al.*, 2004). Recently, Eitas *et al.* (2008) identified TAO1, a TIR-NB-LRR protein that contributes to full disease resistance against *Pto* DC3000 (*avrB*), but not to *Pto* DC3000 (*avrRpm1*). TAO1 is required for AvrB-induced chlorosis in *rpm1* plants, and its activation resulted in *PR1* expression. In this study, the up-regulation of PIA1 by AvrRpm1, but not AvrB, serves as additional evidence for the existence of separate signaling pathways activated by AvrB and AvrRpm1.

The challenge of finding PIA1 targets

Due to its rapid appearance, we speculated that PIA1 might be involved in early signaling, and showed that it controls defense responses to AvrRpm1. Although also detectable in microsomal preparations (Figure 1b), PIA1 does not possess membrane-targeting motifs, so it is not certain whether it is truly membrane-localized or membrane-associated. Accordingly, loss of *PIA1* did not influence phosphorylation of two membrane-associated proteins, RIN4 or Remorin (Figure 9), that are associated with RPM1 function. PIA1 lacks putative MAPK interaction motifs and is unlikely to regulate MAPK activation. In a pilot experiment to identify PIA1 targets by two-dimensional electrophoresis, only three potential, probably indirect, candidates (including ATPB, the β -subunit of ATP synthase) with low spot intensities were found (see legends to Figures S3 and S4). Thus, a future challenge will be to identify PIA1 targets that affect the defense signaling reported here.

EXPERIMENTAL PROCEDURES

Plant material, growth and treatment

Arabidopsis thaliana plants were grown under short-day conditions. The transgenic *Arabidopsis* lines used here expressed *avrRpm1* under the control of a dexamethasone-responsive promoter in an *RPM1* (Mackey *et al.*, 2002) or *rpm1-3* background (Grant *et al.*, 1995). To induce *avrRpm1* expression, 4-week-old plants were sprayed with 20 μ M dexamethasone containing 0.0075% Silwet L-77 (Surfactant). The T-DNA insertion alleles of *pia1* were isolated from the SALK collection using standard protocols (<http://signal.salk.edu>).

Pto DC3000, *Pto* DC3000 (*hrpA*) (Roine *et al.*, 1997), *Pto* DC3000 (*avrRpt2*), *Pto* DC3000 (*avrB*) and *Pto* DC3000 (*avrRpm1*) (Bisgrove *et al.*, 1994; Grant *et al.*, 1995) were grown in LB medium with the appropriate antibiotics.

For bacterial growth assays, bacteria were re-suspended to 10^5 cfu ml⁻¹ in 10 mM MgCl₂. Four-week-old plants were syringe-infiltrated, and the number of colony-forming-units was determined at 1 h (taken as day 0), 2 and 4 days after infiltration. Leaf discs from two independent plants were homogenized in 1 ml of sterile water using a Precellys bead beater (Bertin Technologies, <http://www.precellys.fr>). Serial dilutions were used to determine the number of the bacteria.

For all other experiments (*PIA1* and *AtREM1.2* induction, RIN4 phosphorylation, HR and oxidative burst assays, RT-PCR analysis, and JA/OPDA, ET and SA measurements), bacteria were infiltrated at 10^8 cfu ml⁻¹ in 10 mM MgCl₂. flg22 (Felix *et al.*, 1999) was infiltrated at 10 μ M.

Protein analysis

Total protein, microsomal protein, Rubisco-depleted total protein and Rubisco-depleted microsomal protein preparations, two-dimensional PAGE, image analysis and protein identification were performed as described by Widjaja *et al.* (2009).

For Western blot analysis, total protein extracts were prepared by grinding leaf material (100 mg) in 200 μ l of extraction buffer containing 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 5 mM DTT and plant protease inhibitor cocktails. Insoluble debris was removed by centrifugation at 20 000 g for 10 min at 4°C. Protein concentration was determined using a 2D Quant Kit (GE Healthcare, <http://www.gelifesciences.com>) according to the manufacturer's instructions. Total protein (30 μ g) was separated by 12% SDS-PAGE gel and transferred to a nitrocellulose membrane (Hybond-ECL; Amersham Biosciences, <http://www5.amershambiosciences.com/>). The blot was incubated in TBST (140 mM NaCl, 0.1% Tween-20, 20 mM Tris/HCl pH 7.6) containing 5% skimmed milk at 4°C overnight. The blots were incubated for 1 h at room temperature with anti-RIN4 at a dilution of 1:5000 (Mackey *et al.*, 2002) and subsequently with anti-rabbit HRP at a dilution of 1:5000 (Pierce Biotechnology, <http://www.pierce.net.com>) with washing in TBST between incubations and after the secondary antibody step. The blot was developed using ECL Plus (GE Healthcare).

RT-PCR analysis

Total RNA from leaves was isolated using the Trizol method, and reverse transcription was performed as described for the Fermentas First Strand cDNA Synthesis Kit (Fermentas, <http://www.fermentas.com>). PCR was performed with 10% of the cDNA products using the primer combinations and conditions detailed in the Table S1.

Staining

The method used for trypan blue staining was modified from that described by Peterhänzel *et al.* (1997). Leaves were boiled in trypan blue solution (0.033% trypan blue, 8% lactate, 8% glycerol, 8% phenol, 8% water, 67% ethanol) until the green color disappeared. After boiling, the leaves were washed with water, and transferred to saturated chloral hydrate solution (2.5 g chloral hydrate in 1 ml of water) to remove non-specific staining.

The method used for DAB staining was modified from that described by Thordal-Christensen *et al.* (1997). Leaves were incubated in freshly prepared DAB solution (3,3'-diaminobenzidine, 1 mg ml⁻¹, pH 3.8, adjusted with HCl) for 2 h, and then boiled with ethanol to remove the chlorophyll. Further destaining with chloral hydrate was performed as described above.

Hormone analysis

JA and *OPDA*. Ground leaf material (0.5 g) was extracted with 10 ml of methanol containing 100 ng of deuterated JA and OPDA each, as internal standards. Purification and separation by HPLC and quantification by GC-MS were performed as described previously (Stenzel *et al.*, 2003).

SA. Ground leaf material (0.5 g) was assayed for SA and glucose-conjugated SA (SAG) content as described previously (Halim *et al.*, 2007).

ET. ET emissions were measured using a photo-acoustic spectrometer (INVIVO GmbH, <http://www.invivo-gmbh.de>) as described previously (von Dahl *et al.*, 2007). Leaves of *Arabidopsis* were treated as indicated and removed. Excised leaves were transferred to 250 ml cuvettes, and ET was allowed to accumulate in the headspace for 6 h.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Peptide mass fingerprint identification of the PP2C At2g20630 by MALDI-TOF MS.

Figure S2. HR and ROS formation in Col-0 and the *pia1* mutants after *Pto* DC3000 (*avrRpm1*) infiltration.

Figure S3. Screening for putative PIA1 targets by two-dimensional electrophoresis-based proteomics analysis of the *pia1* mutants.

Figure S4. LC-MS/MS identification of ATPB.

Table S1. Primer combinations for gene expression analysis using RT-PCR.

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