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Apoplastic peroxidases are required for salicylic acid-mediated defense against *Pseudomonas syringae*

Nicole D. Mammarella [a,b,1], Zhenyu Cheng [a,b], Zheng Qing Fu [c,2], Arsalan Daudi [d,3], G. Paul Bolwell [d,†], Xinnian Dong [c], Frederick M. Ausubel [a,b,*]

[a] Department of Genetics, Harvard Medical School, Boston, MA 02115, USA
[b] Department of Molecular Biology, Massachusetts General Hospital, Boston, MA 02114, USA
[c] Howard Hughes Medical Institute-Gordon and Betty Moore Foundation, Department of Biology, Duke University, Durham, NC 27708, USA
[d] School of Biological Sciences, Royal Holloway, University of London, Egham, Surrey TW20 0EX, UK

This paper forms part of a special issue of *Phytochemistry* dedicated to the memory and legacy of Professor (Godfrey) Paul Bolwell, MA DSc (Oxon). (1946–2012), internationally-recognised plant biochemist and Regional Editor of *Phytochemistry* (2004–2012). He is much missed by his friends.

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ABSTRACT

Reactive oxygen species (ROS) generated by NADPH oxidases or apoplastic peroxidases play an important role in the plant defense response. Diminished expression of at least two *Arabidopsis thaliana* peroxidase encoding genes, *PRX33* (*At3g49110*) and *PRX34* (*At3g49120*), as a consequence of anti-sense expression of a heterologous French bean peroxidase gene (*asFBP1.1*), were previously shown to result in reduced levels of ROS following pathogen attack, enhanced susceptibility to a variety of bacterial and fungal pathogens, and reduced levels of callose production and defense-related gene expression in response to the microbe associated molecular pattern (MAMP) molecules flg22 and elf26. These data demonstrated that the peroxidase-dependent oxidative burst plays an important role in the elicitation of pattern-triggered immunity (PTI). Further work reported in this paper, however, shows that *asFBP1.1* antisense plants are not impaired in all PTI-associated responses. For example, some but not all flg22-elicited genes are induced to lower levels by flg22 in *asFPB1.1*, and callose deposition in *asFPB1.1* is similar to wild-type following infiltration with a *Pseudomonas syringae hrcC* mutant or with non-host *P. syringae* pathovars. Moreover, *asFPB1.1* plants did not exhibit any apparent defect in their ability to mount a hypersensitive response (HR). On the other hand, salicylic acid (SA)-mediated activation of *PR1* was dramatically impaired in *asFPB1.1* plants. In addition, *P. syringae*-elicited expression of many genes known to be SA-dependent was significantly reduced in *asFBP1.1* plants. Consistent with this latter result, in *asFBP1.1* plants the key regulator of SA-mediated responses, NPR1, showed both dramatically decreased total protein abundance and a failure to monomerize, which is required for its translocation into the nucleus.

1. Introduction

An oxidative burst, comprised primarily of hydrogen peroxide and superoxide has long been known to play a key role in plant defenses, both in pattern-triggered immunity (PTI) and effector-triggered immunity (ETI) (Bradley et al., 1992; Doke, 1983; Lamb and Dixon, 1997; O'Brien et al., 2012a; Thordal-Christensen, 2003; Torres, 2010). PTI is elicited by highly conserved microbe-associated molecular pattern molecules (MAMPs), such as bacterial flagellin or fungal chitin. ETI is generally elicited by pathogen-encoded virulence effectors that are recognized directly or indirectly by cytoplasmic nucleotide-binding site–leucine-rich repeat (NBS–LRR) receptors, referred to generically as Nod-like receptors

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylate; Col-0, Arabidopsis ecotype Columbia; ET, ethylene; ETI, effector-triggered immunity; FBP1, French bean peroxidase 1; JA, jasmonic acid; MAMP, microbe-associated molecular pattern; NLR, Nod-like receptor; NPR1, non-expresser of PR genes 1; PTI, pattern-triggered immunity; *Pto*, *Pseudomonas syringae* pv. *tomato*; *Psm*, *Pseudomonas syringae* pv. *maculicola*; ROS, reactive oxygen species; SA, salicylic acid.

* Corresponding author at: Department of Molecular Biology, Massachusetts General Hospital, Boston, MA 02114, USA. Tel.: +1 617 726 5969.
E-mail address: ausubel@molbio.mgh.harvard.edu (F.M. Ausubel).
[1] Current address: Nixon Peabody LLP, 100 Summer Street, Boston, MA 02110, USA.
[2] Current address: Department of Biological Sciences, University of South Carolina, Columbia, SC 29208, USA.
[3] Current address: Bio-protocol, P.O. Box 61231, Palo Alto, CA 94306, USA.
[†] Deceased.

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or NLRs (Belkhadir et al., 2004; McHale et al., 2006; Nimchuk et al., 2003). Biochemical, pharmacological, and genetic approaches have been used to implicate both nicotinamide adenine dinucleotide phosphate (NADPH) oxidases and apoplastic peroxidases as the source of reactive oxygen species (ROS) (Bindschedler et al., 2006; Bolwell et al., 2002; Grant et al., 2000; Torres et al., 2002).

In *Arabidopsis thaliana*, NADPH oxidases are encoded by 10 *rboh* genes, and *rbohD* and *rbohF* appear to play key roles in the plant defense response. An *rbohD* mutant exhibited a dramatically decreased oxidative burst and a double *rbohD rbohF* mutant exhibited a reduced hypersensitive response (HR) following inoculation with avirulent *Pseudomonas syringae* strains that elicit ETI (Torres et al., 2002). Although *rbohD*/*rbohF* mutants are strongly affected in mounting an oxidative burst and a HR following pathogen challenge, they exhibited only modest or no phenotypes with respect to enhanced susceptibility to *P. syringae* or a decrease in callose deposition or defense gene induction following elicitation with a variety MAMPs (Chaouch et al., 2012; Daudi et al., 2012; Galletti et al., 2008).

The second enzyme family responsible for generating ROS are Class III apoplastic peroxidases, encoded by 73 genes in Arabidopsis (Welinder et al., 2002) and subsets of the family are upregulated by pathogens, salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) (Almagro et al., 2009). While peroxidases usually catalyze the oxidation of substrates via H_2O_2, some members of this family of pH-dependent enzymes are also capable of generating H_2O_2 in the apoplast (Bolwell et al., 1998; O'Brien et al., 2012a).

Recently, additional enzymes have been reported to be involved in pathogen-elicited oxidative bursts. An aspartate oxidase was shown to be required for the RBOHD-triggered ROS burst in Arabidopsis (Macho et al., 2012), and the photorespiratory enzyme glycolate oxidase was shown to play an important role independently of NADPH oxidases in conferring resistance to and in generating H_2O_2 in response to non-host pathogens in Arabidopsis (Rojas and Mysore, 2012; Rojas et al., 2012).

We previously described Arabidopsis plants, in which antisense expression of a heterologous French bean (*Phaseolus vulgaris*) peroxidase (FBP1) cDNA was used to knock-down expression of at least two Arabidopsis peroxidase genes, *At3g49110* (*PRX33*; previously *PCa*) and *At3g49120* (*PRX34*; previously *PCb*). This line, referred to as "H_4" (Bindschedler et al., 2006) or "*asFBP1.1*" (Daudi et al., 2012), fails to mount an oxidative burst in response to pathogen attack and is strikingly susceptible to infection by both bacterial (*P. syringae*) and fungal (*Botrytis cinerea* and *Erisyphe orontii*) pathogens, even succumbing to opportunistic infections in the greenhouse (Bindschedler et al., 2006). T-DNA insertion lines in which the expression of *PRX33* and *PRX34* are affected were also tested for enhanced susceptibility to *P. syringae*. These T-DNA lines, *prx33:T-DNA* [ecotype Wassilewskija (Ws)] and *prx34:T-DNA* (ecotype Col-0) contain insertions in intron 1 of *PRX33* and the promoter region of *PRX34*, respectively (Passardi et al., 2006), and are most likely hypomorphic mutants. A *prx33:T-DNA*; *prx34:RNAi* double knockdown line was derived from the *prx33:T-DNA* line by transforming it with a *prx34:RNAi* construct (Passardi et al., 2006). The *prx33:T-DNA* and the *prx33:T-DNA*; *prx34:RNAi* lines exhibited enhanced susceptibility to *P. syringae*, but not as much as the *asFBP1.1* line (Daudi et al., 2012). The *prx34:T-DNA* was not more susceptible to *P. syringae*. The fact that the *pxr33* and *prx34* T-DNA lines are not as susceptible to *P. syringae* as *asFBP1.1* is most likely a consequence of their hypomorphic nature.

Further investigation of *asFBP1.1* as well as the *prx33* and *prx34* T-DNA insertion mutants (Daudi et al., 2012) and Arabidopsis tissue culture lines in which the expression of *PRX33* and *PRX34* were knocked down by heterologous expression of anti-sense *FBP1* cDNA (O'Brien et al., 2012b), demonstrated that all of these *PRX33*/*PRX34* knockdown lines are at least partially compromised in PTI. That is, wild-type levels of both peroxidases appear to be necessary for full induction of the oxidative burst and upregulation of several defense-related genes elicited by a number of different MAMPs, including the synthetic peptide elicitors flg22 and elf26, which correspond to active epitopes of flagellin and elongation factor Tu, respectively.

While *PRX33* and *PRX34* appear to be an integral part of an effective defense, including PTI, against a number of pathogens, it remains unclear how they mediate resistance. The wide spectrum of pathogens to which *PRX33*/*PRX34* knockdown plants are susceptible suggests that the broad-spectrum, basal defenses associated with PTI or with salicylic acid-associated resistance are dependent upon peroxidase-generated ROS. Additionally, In *Capsicum annum*, the Class III peroxidase CaPrx02 was found to be necessary for the HR that is elicited after infection with the bacterial pathogen *Xanthomonas campestris* pv. *vesicatoria* (Choi et al., 2007), suggesting a role for peroxidases in ETI. In this paper, we further explore the role of peroxidases in promoting resistance to pathogen attack. Here we focus specifically on the *asFBP1.1* anti-sense line because as described above it is more susceptible to infection by *P. syringae* than the *prx33* and *prx34* T-DNA insertion lines (Daudi et al., 2012). We find that a salicylic acid-associated pathway that mediates resistance to bacterial pathogens is severely compromised in *asFBP1.1* plants.

2. Results

2.1. asFBP1.1 plants do not exhibit enhanced susceptibility to a P. syringae hrcC mutant or non-host P. syringae pathovars

As previously reported, in mature Arabidopsis plants, knockdown of *PRX33* and *PRX34* expression can lead to enhanced susceptibility to a wide variety of pathogens, including *P. syringae* pv. *tomato* strain DC3000 (*Pto* DC3000) and *P. syringae* pv. *maculicola* strain ES4326 (*Psm* ES4326) (Bindschedler et al., 2006; Daudi et al., 2012). These experiments involved the ecotype Columbia (Col-0)-derived line (*asFBP1.1*) in which expression of *PRX33* and *PRX34* (and potentially other peroxidases) was knocked down by anti-sense expression of a heterologous French bean cDNA (*FBP1*) encoding a cell wall peroxidase (Bindschedler et al., 2006; Daudi et al., 2012).

The enhanced susceptibility of *asFBP1.1* to *Pto* DC3000 led us to hypothesize that it would also be more susceptible to a *Pto* DC3000 *hrcC* mutant. Because a *hrcC* null mutant lacks expression of one of the proteins required for assembly of the Type III secretion system and is thus incapable of delivering any Type III-secreted effectors to host cells (Roine et al., 1997; Yuan and He, 1996), it should only trigger PTI-associated responses. Furthermore, without the functionality of secreted Type III effectors, a *hrcC* mutant should be incapable of suppressing PTI responses. Thus, in theory, a *hrcC* mutant can be used to determine the role of PTI in conferring resistance during infection independently of ETI. Because the *asFBP1.1* line (Daudi et al., 2012), as well as Arabidopsis tissue culture cells expressing anti-sense French bean peroxidase 1 cDNA (O'Brien et al., 2012b), are compromised for a variety of PTI-related responses, including an oxidative burst, callose deposition, and upregulation of several genes involved in glucosinolate and camalexin biosynthesis in response to several well-characterized MAMPs, we were surprised to find that *asFPB1.1* plants are not more susceptible to *Pto* DC3000 *hrcC* than the wild-type Col-0 control plants (Fig. 1).

We continued investigating the role of *PRX33*/*PRX34* in conferring resistance to *P. syringae* by testing a variety of so-called non-host *P. syringae* pathovars that are not normally pathogenic on Arabidopsis Col-0 plants. Four non-host pathovars of *P. syringae*

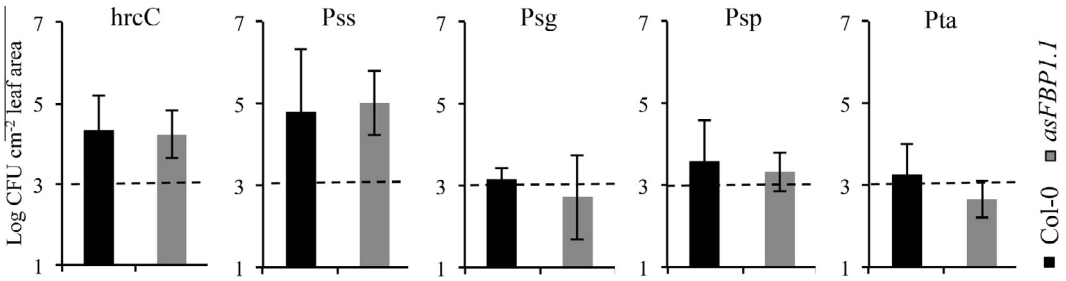

Fig. 1. Growth of *Pto* DC3000 *hrcC* or non-host *P. syringae* pathovars is not enhanced in *asFBP1.1*. As described in Section 4, four-week old Arabidopsis leaves were syringe-infiltrated with the specified strains at a dose of approximately 1×10^3 CFU per cm^2 leaf area, leaves were harvested at 5 days after infiltration, and bacterial titers were determined. Data represent the mean of bacterial titers ± S.D. of ten leaf disks excised from 10 leaves of 5 plants. Growth of *Pto* DC3000 *hrcC* or the non-host pathovars was not significantly different in *asFPB1.1* plants than in the Col-0 control plants as determined by a Student's *t* test (*p* value of <0.05). The infection assay was repeated three times with similar results. hrcC, *Pto* DC3000 *hrcC*; Pss, *P. syringae* pv. *syringae* B728a; Psg, *P. syringae* pv. *glycinea* race 4; Psp, *P. syringae* pv. *phaseolicola* NPS 3121; Pta, *P. syringae* pv. *tabaci* 11528.

that do not trigger a visible HR that is associated with ETI were used to infect *asFBP1.1* and wild-type Col-0 plants (Fig. 1). Similarly to the results obtained with the *Pto* DC3000 *hrcC* mutant, *asFBP1.1* did not exhibit enhanced susceptibility to any of the non-host *P. syringae* pathovars. Once again, assuming that non-host resistance is mediated at least in part by PTI, and given the fact that *asFBP1.1* is compromised for PTI-related responses triggered by individual MAMPs (Daudi et al., 2012), we were surprised to find that the peroxidase compromised plants were not more susceptible to the non-host *P. syringae* pathovars.

2.2. Callose deposition is not impaired in asFBP1.1 during infection with a P. syringae hrcC mutant or non-host P. syringae pathovars

Whereas *asFBP1.1* is impaired for an oxidative burst, callose deposition, and expression of glucosinolate and camalexin biosynthetic genes following elicitation with a variety of individual MAMPs (Daudi et al., 2012; O'Brien et al., 2012b), as shown in the previous section, these deficiencies in MAMP signaling in *asFBP1.1* do not appear to compromise resistance triggered by *Pto* DC3000 *hrcC* or various non-host *P. syringae* pathovars. These data suggest that either *Pto* DC3000 *hrcC* and the non-host pathovars are capable of eliciting a strong PTI response in *asFBP1.1* or that the PTI response is not important in conferring resistance to *Pto* DC3000 *hrcC* or to the non-host pathovars. To help distinguish these possibilities, we examined callose deposition following infiltration with *Pto* DC3000 *hrcC* and four non-host *P. syringae* pathovars (Fig. 2). As expected *Pto* DC3000 did not elicit callose formation in the Col-0 control or in *asFBP1.1*, since it is well established that *Pto* DC3000 type III effectors block MAMP-mediated signaling (Block and Alfano, 2011; Hauck et al., 2003) (Fig. 2). In contrast to wild-type *Pto* DC3000, *Pto* DC3000 *hrcC*, as well as the four non-host *P. syringae* pathovars, elicited a strong callose deposition response in wild-type Col-0 (Fig. 2). However, *Pto* DC3000 *hrcC* and the non-host pathovars also elicited a strong callose response in *asFBP1.1* plants (Fig. 2).

To test whether the callose deposition elicited by the *hrcC* mutant was caused by wounding or other active processes that required live bacteria, heat-killed *Pto* DC3000 *hrcC* was infiltrated into mature leaves. Although the plants appeared to accumulate less callose when challenged with heat-killed *Pto* DC3000 *hrcC* than live *Pto* DC3000 *hrcC* mutant, the level of callose detected in wild-type plants and *asFBP1.1* plants was comparable (Fig. 2).

As stated above, we previously showed that several MAMPs failed to elicit callose deposition in the peroxidase knockdown lines at concentrations that elicited callose in wild type plants. However, we also showed that high concentrations of flg22 were capable of eliciting callose deposition in *asFBP1.1* (Daudi et al., 2012), suggesting that *asFBP1.1* is not a complete null with respect to peroxidase expression or that other pathways leading to callose formation that do not involve the peroxidases can be activated by high concentrations of elicitor. To determine whether heat-killed *Pto* DC3000 *hrcC* activated callose deposition in *asFBP1.1* as shown in Fig. 2 solely because it was infiltrated at a high concentration, we also infiltrated heat-killed *Pto* DC3000 *hrcC* into *asFBP1.1* at a variety of doses. At an OD_{600} = 0.01, callose deposition in wild-type plants begins to decrease notably. At all concentrations, however, *asFBP1.1* plants displayed the same amount of callose deposition as Col-0 wild-type plants (Supplementary Fig. 1), suggesting that *asFBP1.1* is equally capable to respond to the heat-killed cells as wild-type plants.

The data in this section indicate that although *PRX33*/*PRX34* knockdown plants are compromised in PTI responses when elicited with individual MAMPs, they do not appear to be substantially compromised in PTI responses when infiltrated with live or heat-killed pathogens.

2.3. Defense gene activation in asFBP1.1

We previously showed that MAMP-mediated activation of genes involved in indole glucosinolate and camalexin biosynthesis is compromised in *PRX33*/*PRX34* knockdown plants and cell cultures (Daudi et al., 2012; O'Brien et al., 2012b). To further examine defense-related gene activation in *asFBP1.1*, we sought to determine whether the expression of genes associated with ethylene signaling (*ERF1*), ethylene and jasmonic acid signaling (*PR3*, *PR4*), and salicylic acid signaling (*GST6*) is affected in *asFBP1.1*. The *asFBP1.1* line was infiltrated with flg22 and induction of the defense-associated genes was measured by qRT-PCR. Similarly to the PTI-associated genes that we previously examined (*CYP71A12*, *CYP79B2*, *CYP81F2*, and *MYB51*; (Daudi et al., 2012)), 1 μM flg22-elicited activation of *ERF1* and *GST6* was significantly attenuated in *asFBP1.1* (Fig. 3). In contrast, 1 μM flg22-mediated activation of *PR3* and *PR4* was less affected in *asFBP1.1* plants (Fig. 3). These data support the conclusion that *asFBP1.1* is compromised for some but not all PTI-related responses and provide an explanation for the observation that *asFBP1.1* does not exhibit enhanced susceptibility to *Pto* DC3000 *hrcC* or non-host *P. syringae* pathovars (Fig. 1) and does not exhibit a severely diminished callose deposition response to *Pto* DC3000 *hrcC* and non-host pathovars (Fig. 2).

2.4. asFBP1.1 is not impaired in ETI

To further probe the specific deficits in the defense response in *asFBP1.1* that make it more susceptible to some pathogens, we

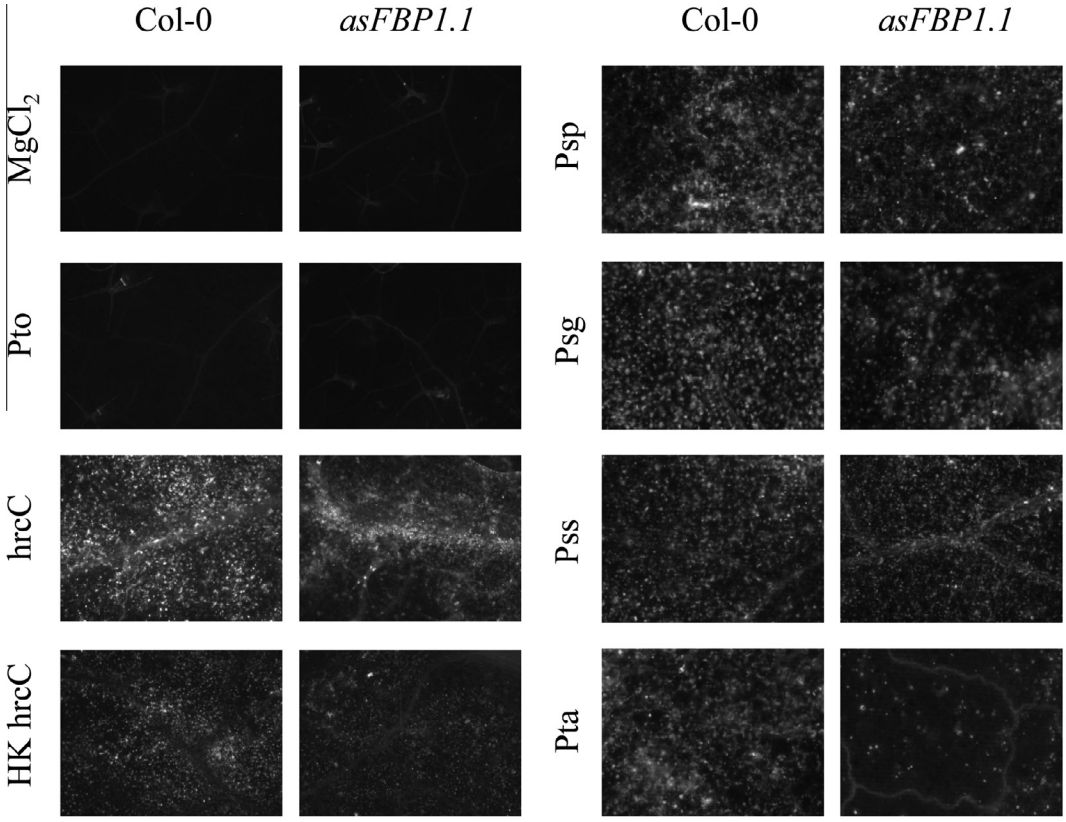

Fig. 2. *Pto* DC3000 *hrcC* or non-host *P. syringae* pathovars elicits similar levels of callose accumulation in the leaves of Col-0 and *asFBP1.1* plants. Approximately 0.1 mL of *Pto* DC3000, DC3000 *hrcC*, heat-killed DC3000 *hrcC*, or the non-host pathovars at OD_{600} = 0.2 were infiltrated into the rosette leaves of four-week old Arabidopsis plants. At least 3 independent plants were used as biological replicates and 6 rosette leaves were sampled from each plant. The experiment was repeated 3 times. Representative leaves are shown. Infiltrated plants were incubated 16–20 h and then leaves were harvested and stained for callose as described in Section 4. Pto, *P. syringae* pv. *tomato* strain DC3000; hrcC, *Pto* DC3000 *hrcC*; HK hrcC, heat-killed *Pto* DC3000 *hrcC*; Pss, *P. syringae* pv. *syringae* B728a; Psg, *P. syringae* pv. *glycinea* race 4; Psp, *P. syringae* pv. *phaseolicola* NPS 3121; Pta, *P. syringae* pv. *tabaci* 11528.

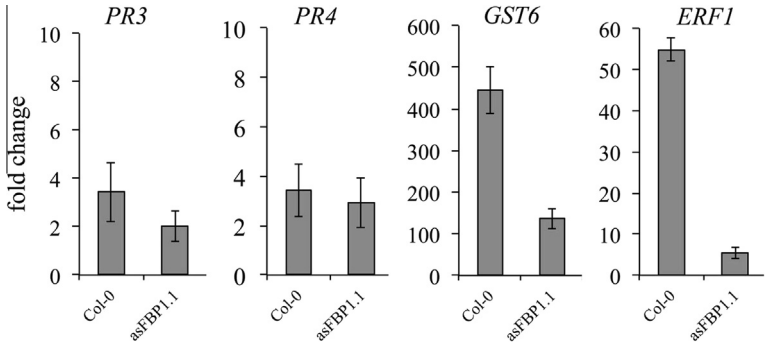

Fig. 3. qRT-PCR analysis of flg22-elicited genes in wild-type and *asFBP1.1* plants. Quantitative RT-PCR analysis of *PR3*, *PR4*, *GST6* and *ERF1* transcripts in four-week old rosette leaves 6 h following infiltration of 1 μM flg22. Data represent the average ± standard deviation; *n* = 3 replicate samples, each containing approximately 6 leaves of 3 plants. The experiment was repeated 3 times with similar results. *ERF1* and *GST6* transcript levels were significantly lower in the *asFBP1.1* line compared to Col-0, whereas *PR3* and *PR4* levels were not.

investigated the potential role that peroxidases play in ETI. Specifically, Col-0 wild-type or *asFBP1.1* plants were infiltrated with *Pto* DC3000 or *Pto* DC3000 expressing one of five Type III effectors (*avrRpm1*, *avrRps2*, *avrB*, *avrRps4*, or *avrPphB*), all of which elicit an HR response in the Col-0 ecotype (Fig. 4 and Supplementary Fig. 2). As expected, *Pto* DC3000 grew to significantly higher titers in the leaves of *asFBP1.1* plants than in wild-type plants. Similarly, *Pto* DC3000 carrying any of the five effectors grew to higher titers in *asFBP1.1* than in the wild-type plants. However, in no case did the avirulent strains (expressing the effectors) grow to as high a titer in *asFBP1.1* as *Pto* DC3000 not expressing an effector. These data suggest that in all cases, the type III effectors elicit a significant defense response in the *asFBP1.1* peroxidase knockdown line. Similar results were obtained when Col-0 and *asFBP1.1* plants were infiltrated with the less virulent strain *Psm* ES4326 or with ES4326 expressing *avrRpm1* or *avrRpt2* (Fig. 4).

We sought to confirm the data in Fig. 4 and Supplementary Fig. 2 by testing whether *PRX33* and *PRX34* are required for elicitation of the HR itself. Mature leaves of Col-0 and *asFBP1.1* plants were infiltrated with *Pto* DC3000 expressing *avrRpt2* or *avrRpm1* and stained for cell death using trypan blue. As negative controls, plants were infiltrated with water, *Pto* DC3000, or *Pto* DC3000 *hrcC*. As shown in Fig. 5A, water, *Pto* DC3000 or *Pto* DC3000 *hrcC* did not elicit a significant amount of cell death in either wild-type plants

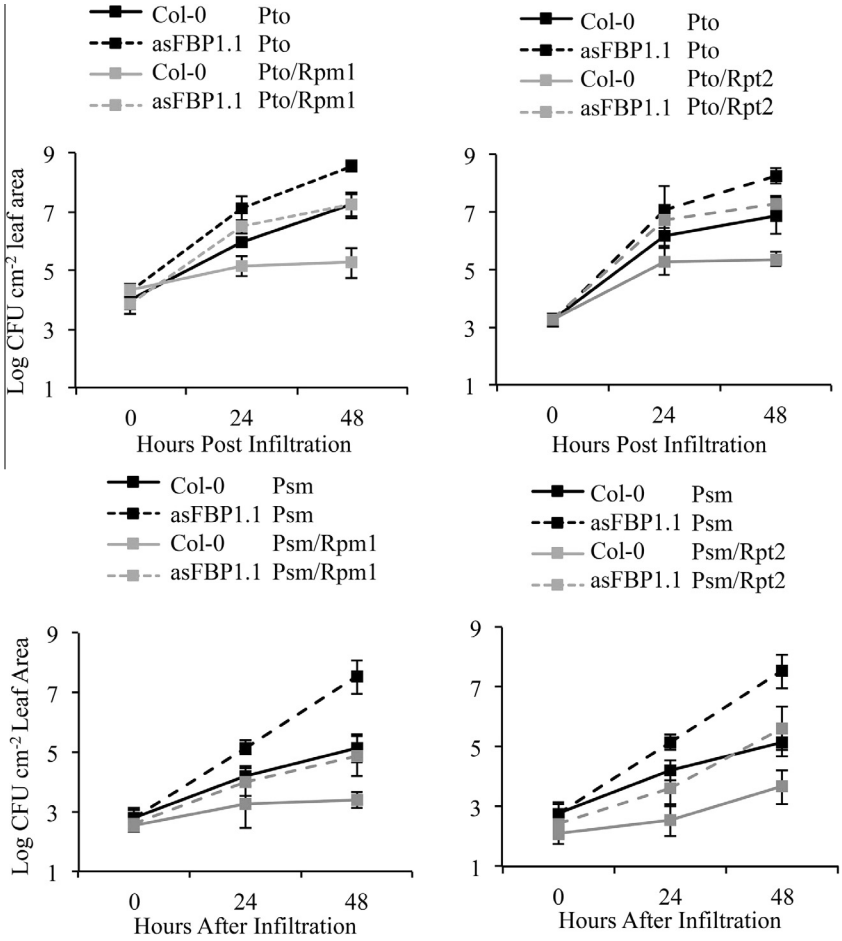

Fig. 4. *PRX33* and *PRX34* are not required for ETI. As described in Experimental, four-week old Col-0 or *asFBP1.1* leaves were syringe-infiltrated with the specified strains at a dose of approximately 1.0×10^3 CFU per cm^2 leaf area, leaves were harvested at 0, 24, and 48 h after infiltration, and bacterial titers were determined. Data represent the mean of bacterial titers ± S.D. of ten leaf disks excised from 10 leaves of 5 plants. The infection assay was repeated three times with similar results. Pto, *Pto* DC3000; Pto/Rpm1 or Pto/Rpt2, *Pto* DC3000 expressing *avrRpm1* or *avrRpt2*; Psm, *Psm* ES4326; Psm/Rpm1 or Psm/Rpt2, *Psm* ES4326 expressing *avrRpm1* or *avrRpt2*.

or *asFBP1.1* plants. However *Pto* DC3000 carrying either *avrRpt2* or *avrRpm1* elicited extensive cell death in both wild-type and *asFBP1.1* leaves, suggesting that the peroxidase knockdown line is capable of mounting a robust HR.

As an independent measure of an HR, we also determined the extent of ion leakage. Mature Col-0 or *asFBP1.1* leaves were infiltrated with a water control, *Pto* DC3000, or *Pto* DC3000 expressing *avrRpm1*, and ion leakage caused by cell death was measured with a conductivity meter (Fig. 5B). *Pto* DC3000 expressing *avrRpm1* elicited significantly more ion leakage than *Pto* DC3000 but the amount of ion leakage in response to *Pto* DC3000 *avrRpm1* was statistically indistinguishable in wild-type and *asFBP1.1* plants.

2.5. Peroxidases play an essential role in SA signaling

We next tested whether *asFBP1.1* plants respond aberrantly to the defense-related hormones jasmonic acid (JA), ethylene (ET), or salicylic acid (SA). This was examined by spraying mature plants with the hormones [1-aminocyclopropane-1-carboxylate (ACC) in the case of ET] and assaying the responsiveness of the hormone-signaling pathway by measuring the activation of selected hormone-responsive genes using qRT-PCR (Fig. 6 and Supplementary Fig. 3). These experiments showed that *asFPB1.1* plants were severely compromised in the induction of the SA-responsive genes *PR1*, *GST6*, and *PR2* after SA treatment (Fig. 6). In contrast to the results obtained with the SA-responsive genes, the JA-responsive genes *VSP1*, *VSP2*, *PDF1.2*, *PR3*, and *PR4* and the ET-responsive genes *PR3*, *PR4*, *ERF1*, and *MYB51* were activated at least to the same levels by JA or ET, respectively, in wild-type Col-0 and *asFBP1.1* plants (Supplementary Fig. 3). In some experiments, the *VSP1* and *VSP2* genes were activated to higher levels in *asFBP1.1* plants than in wild-type. This latter result may suggest that the higher level of JA-mediated signaling in *asFPB1.1* might be accompanied by a concomitant decrease in SA signaling as a consequence of the well-established phenomenon of SA–JA antagonism (Mur et al., 2006; Spoel et al., 2003). In general, however, these data suggested that *asFBP1.1* plants are significantly impaired in SA-signaling but are most likely not impaired in JA or ET signaling pathways.

To confirm and extend the results described above (in Fig. 6) with SA-responsive genes, we measured the expression of a panel of 30 selected genes related to SA signaling in Col-0 and *asFBP1.1* plants using nanoString technology that involves the use of fluorescent barcoded probes that hybridize to mRNAs to monitor their relative levels (Geiss et al., 2008). We measured both the basal levels of these genes and the levels following infection with *Pto* DC3000 or *Pto* DC3000 expressing *avrRpm1*. We found that the basal levels of 15 of these 30 selected genes were significantly lower in *asFBP1.1* plants than in wild-type plants (Table 1). The data for four selected SA-responsive genes is plotted in Fig. 7. In addition, the basal levels of *PR2* and *PR5* in *asFBP1.1* plants were less than 20% of their basal levels in wild-type plants, even though the *p* values were slightly higher than 0.05. Importantly, when infiltrated with *Pto* DC3000 or *Pto* DC3000 expressing *avrRpm1*, seven out of the 15 basally suppressed genes also showed signifi-

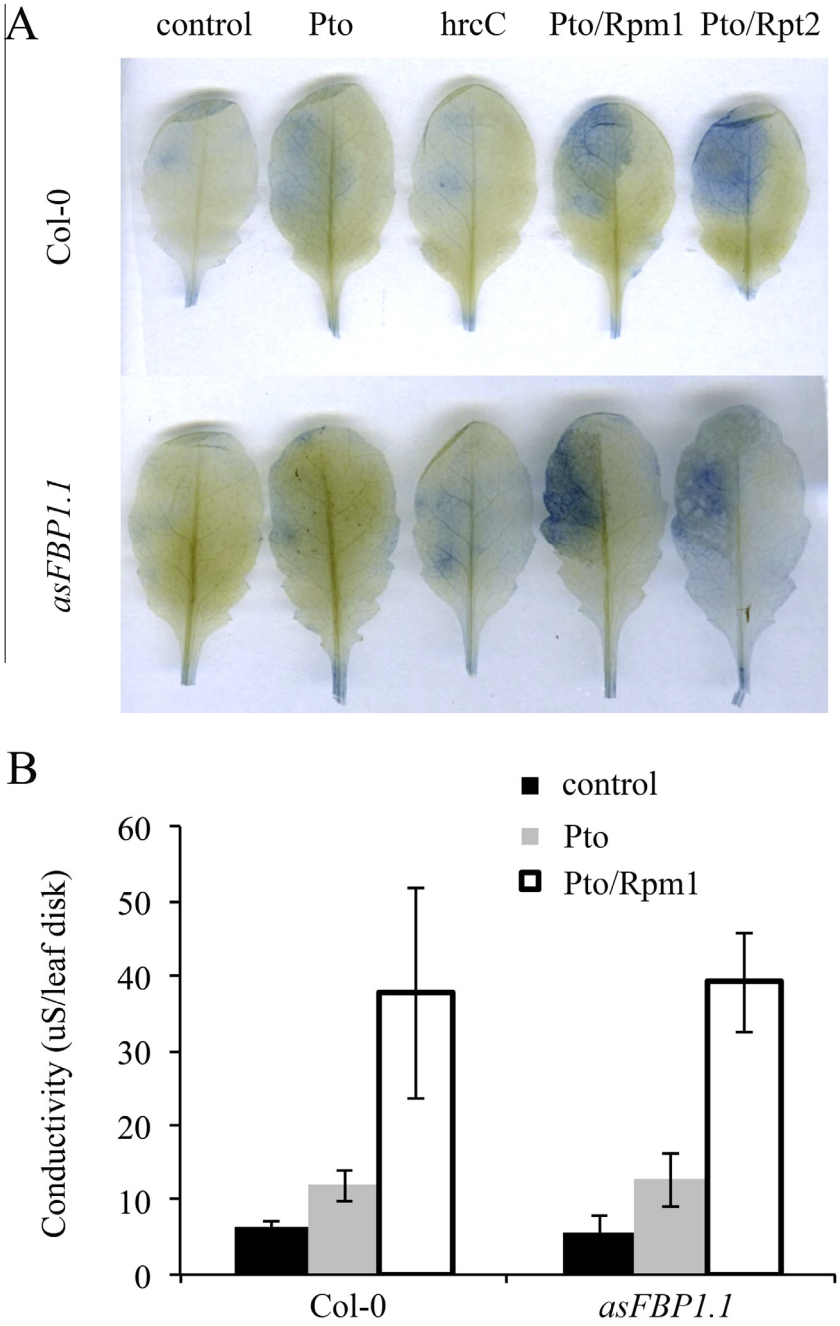

Fig. 5. Cell death is not compromised in *asFBP1.1*. (A) Trypan blue staining carried out as described in Section 4 of leaves of four-week old Col-0 or *asFBP1.1* plants infected with mock control, *Pto* DC3000 (Pto), *Pto* DC3000 *hrcC* mutant (hrcC), or *Pto* DC3000 expressing *avrRpm1* (Pto/Rpm1) or *avrRpt2* (Pto/Rpt2). (B) Ion leakage carried as described in Section 4 of four-week old Col-0 or *asFBP1.1* leaves infected with mock control, *Pto* DC3000 (Pto), or *Pto* DC3000 expressing *avrRpm1* (Pto/Rpm1). The experiments in (A) and (B) were repeated 3 times with similar results.

cantly abrogated activation in *asFBP1.1* plants compared to wild-type plants (Fig. 7 and Table 1), showing that the SA-mediated signaling pathway is significantly impaired in *asFBP1.1* plants, a likely cause of their enhanced susceptibility to pathogenic infections. Interestingly, the salicylic acid synthetic gene *ICS1*(*SID2*) was significantly upregulated in *asFBP1.1* plants during the process of pathogen infection (Table 1), suggesting a positive feedback when SA-signaling is disrupted.

2.6. NPR1 monomer accumulation is impaired in asFBP1.1 plants

Extensive previously published work has shown that when the SA pathway is activated, a change in the redox state of the cell induces NPR1 to monomerize and translocate to the nucleus to promote transcription of SA-responsive genes, including NPR1 itself (Mou et al., 2003; Tada et al., 2008). Reasoning that *asFBP1.1* plants may exhibit altered cellular redox states following SA treatment, we monitored the induction and monomerization of NPR1 by gel electrophoresis followed by western blot analysis using an anti-NPR1 antibody. Fig. 8A shows that in *asFBP1.1* plants, total NPR1 protein accumulates to significantly lower levels than in wild-type plants following elicitation with SA. Strikingly, no NPR1 monomer was detectable in *asFBP1.1* plants following activation of the SA pathway (Fig. 8B).

Interestingly, *PDF1.2*, a JA/ET-regulated gene, appeared to be highly expressed in *asFBP1.1* plants compared to Col-0 (Table 1),

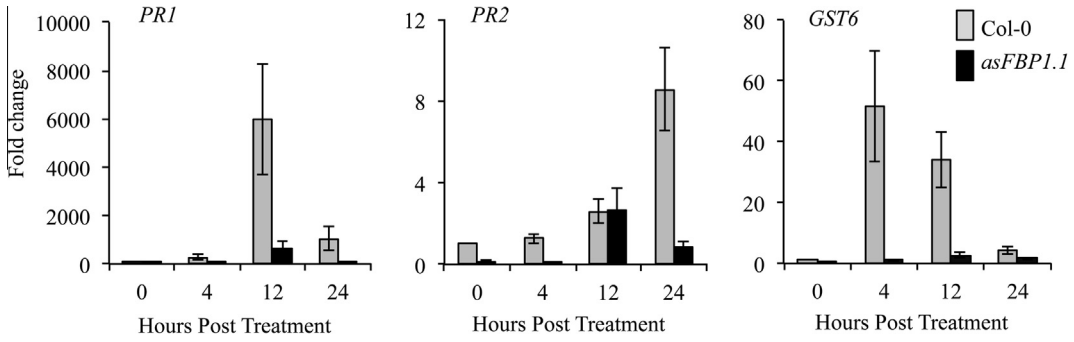

Fig. 6. SA-induced genes expression is disrupted in *asFBP1.1*. qRT-PCR analysis of SA elicited genes in Col-0 and *asFBP1.1*. Four-week old Col-0 or *asFBP1.1* plants were sprayed with 1 mM SA and at the indicated times after treatment, leaves were harvested and analyzed for *PR1*, *PR2*, and *GST6* mRNA accumulation as described in Section 4. Fold induction data represent the mean ± S.D., $n = 3$ replicate samples, each containing approximately 6 leaves of 3 plants. The experiment was repeated 3 times with similar results.

Table 1
Expression analysis of SA response-related genes. Ratio of mRNA abundance in *asFBP1.1* mutant relative to wild-type plants at basal level or 48 h after pathogen infections as determined by nanoString transcriptomic analysis. Data are from three replicate samples.

	Relative ratios *asFBP1.1* Vs Col-0		
SA response-related genes	Basal	*Pto*	*Pto*/Rpm1
BGL2 (*PR-2*)	0.12	0.56	1.42
CBP60G	0.51	1.51	0.96
CBP60H	0.35**	0.87	0.96
CHI	0.17*	0.85	1.52
CHI-B (*PR-3*)	1.41	1.31	1.74
CML41	0.31*	0.75	1.78*
CRT3 (*PSL1*)	0.56**	0.49	0.81*
CYP71B15 (*PAD3*)	0.19	0.96	1.67
CYP71B23	0.29**	0.69**	0.91
EDS1	0.69	0.50*	0.89
GPA1	1.11	1.18	1.39*
ICS1 (*SID2*)	1.24	1.87**	1.86**
LTP inhibitor	0.30**	0.44	1.34
MKK1 (*MEK1*)	0.97	0.67	1.17
MKK2	0.97	0.77	0.96
MLO2 (*PMR2*)	0.86	0.74	0.80
NIMIN1	0.15**	0.40*	0.35*
NPR4	0.97	0.54	0.80
PAD4	0.59**	1.31*	1.13
PCS1 (*PEN4-CAD1*)	0.93	0.78	1.03
PDF1.2	2.32	1.43	11.6
PEN1-SYP121	0.74*	0.93	1.09
PR-1	0.03*	0.28*	0.41
PR-5	0.14	0.47	1.61
TRX3	1.08	1.04	1.12
TRX5	0.49*	0.92	0.85
WAK1	0.30**	0.45	0.97
WRKY38	0.01*	0.37*	0.25**
WRKY54	0.19**	0.31**	0.59*
WRKY70	0.30**	0.49*	0.84

* $p < 0.05$.
** $p < 0.01$, two-tailed *t*-test.

similar to the results obtained with the *VSP1* and *VSP2* genes (Supplementary Fig. 3). Although this result is expected as a consequence of SA/JA antagonism (Mur et al., 2006; Spoel et al., 2003) if SA signaling is impaired in *asFBP1.1* plants, the data did not reach statistical significance.

3. Discussion

In previous publications, we reported that Arabidopsis plants knocked down for the expression of the *PRX33* and *PRX34* cell wall-associated peroxidase genes are more susceptible to a variety of pathogens and exhibit a variety of compromised responses to individual MAMPs, including flg22 and elf26. The plant response to MAMPs is referred to as pattern triggered immunity (PTI) and in this paper we sought to determine whether the compromised PTI phenotype of the peroxidase knockdown line *asFBP1.1* is sufficient to explain their hyper-susceptibility to pathogens. The rationale for this hypothesis is that the breadth of pathogens to which peroxidase-compromised lines are susceptible, including bacteria as well as both necrotrophic and biotrophic fungal pathogens, suggests a general defect in basal and/or non-host defenses. Surprisingly, however, our data show that the hyper-susceptibility of *asFBP1.1* plants is mostly likely due to a defect in the SA-dependent defense response pathway.

Although *asFBP1.1* plants are clearly defective in mounting an oxidative burst, callose deposition, and expression of particular defense-related genes following treatment with individual MAMPs, they exhibit wild-type PTI-associated responses when challenged with heat-killed bacteria, a *P. syringae hrcC* mutant that is defective in Type III secretion, or with a variety of non-host *P. syringae* pathovars. Similarly, the *asFBP1.1* line does not appear to be defective in effector-triggered immunity (ETI), responding like wild-type plants to infection with *P. syringae* expressing a variety of type III effectors. Normal ETI responses in the *asFBP1.1* knockdown line included the ability to mount a hypersensitive cell-death response and to restrict the growth of two different "avirulent" *P. syringae* strains expressing type III effectors. With respect to restricting the growth of avirulent strains, the *asFBP1.1* line has a similar phenotype to *npr1* mutant plants that exhibit an enhanced susceptibility to infection with different pathogens (Cao et al., 1994). That is, although *Pto* DC3000 and *Psm* ES4326 expressing various type III effectors grew to lower titers than the *P. syringae* strains without the effectors, both the virulent and avirulent strains grew to higher titers in *asFBP1.1*, respectively, than in the wild-type Col-0 plants. In previously published work, the *asFBP1.1* line was reported to be defective in ETI when infiltrated with *Psm* ES4326 *avrRpt2* (Bindschedler et al., 2006). However, when these experiments were repeated at lower inoculation titers and bacterial growth was measured at later time points, the original observations reported in the Bindschedler et al. (2006) paper could not be replicated. Because we obtained similar results in the current work with both *Pto* and *Psm* and with five different *avr* genes, we are confident that the *asFBP1.1* line is not significantly impaired in ETI.

Importantly, although *asFBP1.1* plants exhibited wild-type PTI and ETI responses when infected with the selected pathogens (as opposed to being elicited with individual MAMPs), they were markedly impaired in responding to SA and in mounting a variety of SA-mediated responses following infiltration of *P. syringae*. Interestingly, among a set of 30 genes related to SA signaling the basal level of expression of 15 genes, as well as the activated levels following pathogen attack, were reduced in *asFBP1.1*. In contrast to aberrant SA-mediated responses in *asFBP1.1* plants, JA and ET sig-

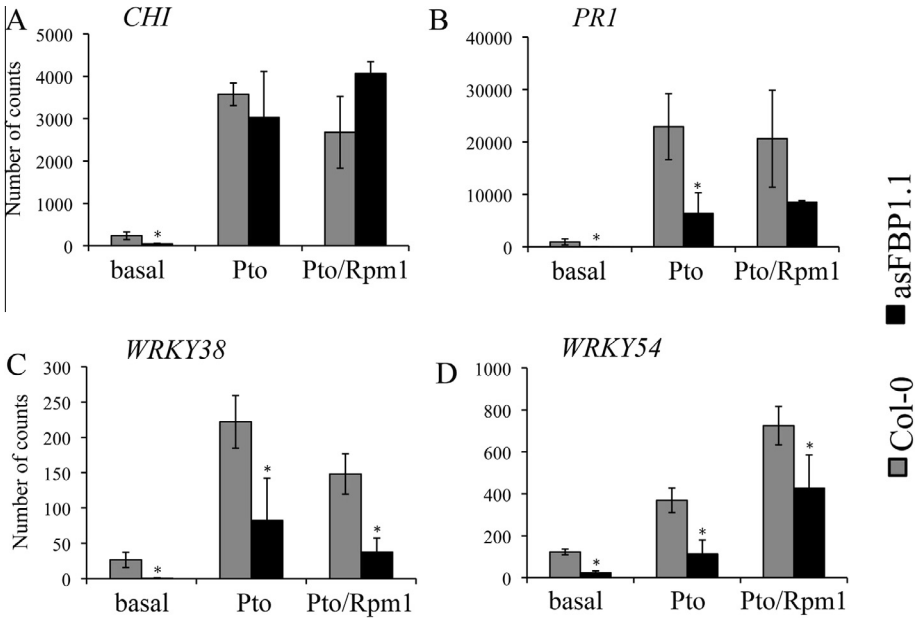

Fig. 7. NanoString analysis of transcript levels of genes involved in SA signaling in *asFBP1.1* plants following infection with virulent and avirulent *P. syringae* strains. As described in Section 4, four-week old Arabidopsis leaves were syringe-infiltrated with *Pto* DC3000 or *Pto* DC3000 expressing *avrRpm1* at a dose of approximately 1.0×10^3 CFU per cm^2 leaf area, leaves were harvested at 72 h after infiltration, and RNA was isolated. RNA samples were hybridized to custom-synthesized gene-specific nanoString barcodes (see Table 1 for a list of the gene transcripts analyzed) and the relative abundance of each gene transcript was measured using the nanoString Analyzer according to the manufacturer's instructions. Compared to Col-0 plants, in the *asFBP1.1* line, *CHI* basal transcript levels were lower (A); *PR1* transcripts were lower at basal and *Pto* DC3000 activated levels (B); and *WRKY38* and *WRKY54* transcripts were lower under all conditions (C and D). Number of counts represents the mean ± S.D., $n = 3$ replicate samples, each containing approximately 6 leaves of 3 plants. $^*p < 0.05$; two-tailed *t*-test.

naling does not appear to be affected. Critically, following SA treatment, NPR1 levels were dramatically lower in *asFB1.1* plants than in wild type. Moreover, following SA treatment, no monomerization of NPR1 could be detected in *asFBP1.1* plants. Although we did not measure SA levels in *asFBP1.1* plants before or after *P. syringae* infection, the levels of *ISC1* (*SID2*) mRNA, which encodes isochorismate synthase, the key enzyme in SA biosynthesis, was modestly but significantly ($p < 0.01$) higher in *P. syringae*-infected *asFPB1.1* plants than in Col-0 plants (Table 1), suggesting that *asFBP1.1* contains at least wild-type levels of SA. Thus, we conclude that the reason the peroxidase mutants exhibit enhanced susceptibility to pathogen attack is primarily due to aberrant SA-mediated signaling.

What is the explanation for the *PRX33*/*PRX34* knockdown lines exhibiting aberrant PTI responses to a variety of individual MAMPs but not to pathogens? The data presented here do not allow us to definitively answer this question. One possibility is that cell wall-associated peroxidases such as PRX33 and PRX34 may be required for signaling downstream of some but not all MAMPs. This seems unlikely, however, since we previously showed that *asFBP1.1* was deficient in eliciting both an oxidative burst and callose deposition in response to four chemically defined MAMPs, flg22, elf26, peptidoglycan, and oligogalacturonides (Daudi et al., 2012) that correspond to three different categories of receptors, leucine-rich repeat receptor like kinases (flg22, elf26; Gomez-Gomez and Boller, 2000; Zipfel et al., 2006), LysM receptor like kinases (peptidoglycan; Willmann et al., 2011; Liu et al., 2012), and wall associated kinase 1 (oligogalacturonides; Brutus et al., 2010). *asFBP1.1* is also deficient in a variety of defense responses after treatment with a crude cell wall preparation from *Fusarium oxysporum* that presumably contains a variety of MAMPs (Daudi et al., 2012). On the other hand, the data presented in this paper show that the peroxidase-generated oxidative burst is required for the activation of some but not all downstream defense-related genes, and in particular that SA-mediated defense-gene activation requires the peroxidases. Our data are consistent with an essential role for cell-wall associated peroxidases in the activation of SA defense pathways that play a major role in conferring pathogen resistance, but not necessarily in the activation of defense responses associated with PTI, such as callose deposition.

The explanation for why *asFPB1.1* does not respond like wild-type to individual MAMPs but does to intact pathogens may simply be a consequence of the fact that *asFPB1.1* behaves as a hypomorphic peroxidase mutant and that simultaneous weak activation of many parallel MAMP signaling pathways generates a sufficient output signal to overcome the defect caused by partial knockdown of *PRX33* and *PRX34* expression. Importantly, as we have discussed in previous publications (Bindschedler et al., 2006; Daudi et al., 2012; O'Brien et al., 2012b), it is very likely that all of the peroxidase knockdown plants that we have examined retain partial expression of the peroxidases, since null mutants are most likely lethal. In any case, during the course of an actual infection, intact pathogens are able to generate a signal that is sufficient to induce PTI-associated responses including callose deposition and resistance to non-host pathogens.

In summary, we have demonstrated that the *asFBP1.1* antisense line exhibits an aberrant SA-dependent response to *P. syringae* infection, which apparently increases susceptibility to both virulent and avirulent *P. syringae* strains. Unexpectedly, we found that even though PTI is significantly impaired in *asFBP1.1* plants in response to individual MAMPs, the PTI response to heat-killed *P. syringae* or to a *P. syringae hrcC* mutant appears to be mostly intact. In addition, ETI elicited by a variety of effectors does not appear to be impaired. The impaired SA-mediated response in *asFBP1.1* explains its enhanced susceptibility to *P. syringae* and to biotrophic fungal pathogens such as powdery mildew. On the other hand, *asFBP1.1* is also highly susceptible to necrotrophic pathogens such as *B. cinerea*, but it is not impaired in jasmonic acid or ethylene signaling. Plants that are defective in SA signaling would be expected to exhibit enhanced JA/ET signaling as a consequence of canonical SA/JA antagonism (Mur et al., 2006; Spoel et al., 2003), which should confer resistance to

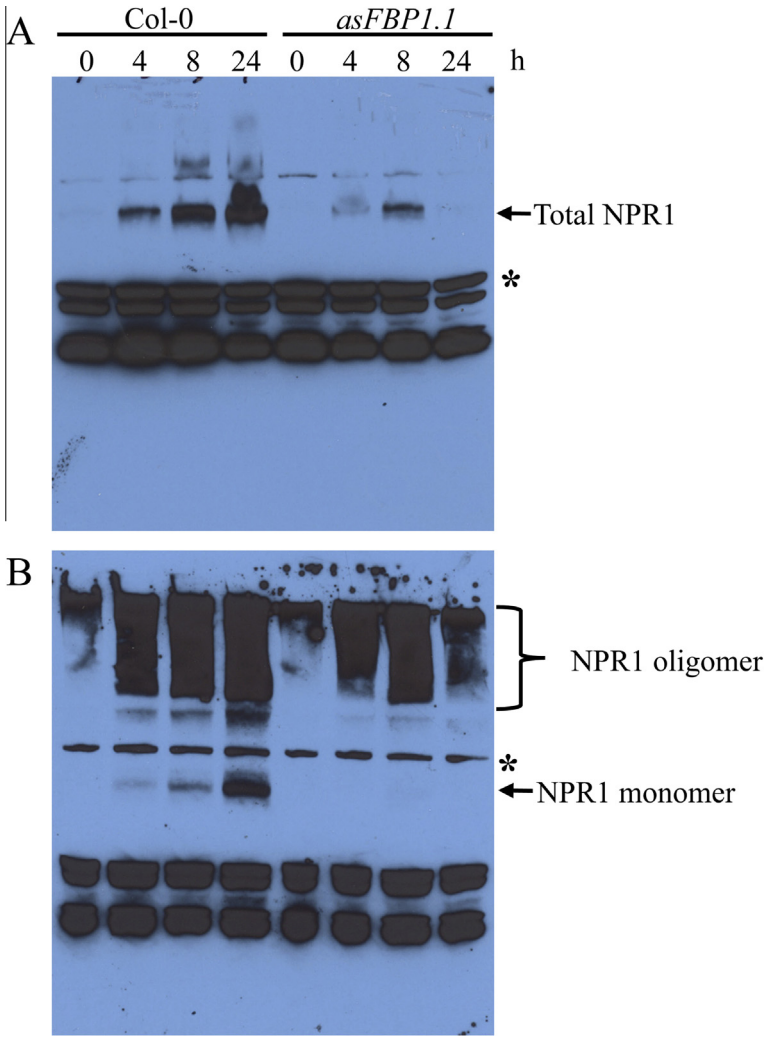

Fig. 8. *asFBP1.1* plants are dramatically reduced in NPR1 total protein and NPR1 monomer formation after SA treatment. Col-0 and *asFBP1.1* plants were sprayed with 0.5 mM salicylic acid. Samples were collected at 0, 4, 8 and 24 h after the treatment. Total protein was isolated and denatured in SDS sample buffer with (A) or without (B) 100 mM DTT, separated on a protein gel, and transferred onto a nitrocellulose membrane. NPR1 total protein (A) and NPR1 monomer (B) were detected using an anti-NPR1 antibody. * Indicates a non-specific band which was used as a loading control in (A) and (B).

necrotrophs. Thus, there is still much to be learned about the role of peroxidases in the plant defense response to pathogen attack.

4. Experimental

4.1. Plants and associated growth conditions

The *asFBP1.1* line used in this study has been described previously (Bindschedler et al., 2006; Daudi et al., 2012). Briefly, *asFBP1.1* is a transgenic line expressing the French bean peroxidase 1 cDNA in an antisense orientation under the control of the CAMV-35S promoter, resulting in the silencing of both *PRX33* and *PRX34*.

Seeds were treated at 4 °C for at least 48 h, but not more than 7 days and then sown on flats of Fafard 2 Mix soil (Conrad Fafard; Agawam, MA) saturated with water containing 4 g of Valent Gnatrol WDG (Walnut Creek, CA) per flat. Plants were reared in chambers set to 12 h days with a daytime temperature of 22 °C and nighttime temperature of 18 °C, 60% RH, and 75 μE.

4.2. Plant treatment with elicitors or hormones

Four-week old plants were infiltrated with 1 μM flg22 as described (Daudi et al., 2012) or sprayed with hormones at the following concentrations unless otherwise specified: 1 mM SA in 0.5% Tween 20; 20 μM MeJA or 50 μM ACC. SA, MeJA and ACC were obtained from Sigma–Aldrich. Samples were harvested 4, 12 and 24 h after SA treatment, or 6 h after JA and ethylene treatment. For elicitor treatments, samples were harvested 6 h after infiltration for qRT-PCR or 18 h after infiltration for callose deposition assays.

4.3. Bacterial strains and infiltrations

The bacterial strains *P. syringae* pv. *tomato* DC3000 (*Pto* DC3000) (Whalen et al., 1991), *P. syringae* pv. *maculicola* ES4326 (*Psm* ES4326) (Dong et al., 1991), *Pto* DC3000 *hrcC* (Yuan and He, 1996), *P. syringae* pv. *syringae* B728a (Feil et al., 2005); *P. syringae* pv. *glycinea* race 4 (Staskawicz et al., 1984), *P. syringae* pv. *phaseolicola* NPS 3121 (Lindgren et al., 1986), and *P. syringae* pv. *tabaci* 11528 (Alfano et al., 1997) have been described, as have the type III effector genes *avrRpt2* (Dong et al., 1991; Whalen et al., 1991), *avrRpm1* (Debener et al., 1991), *avrRps4* (Hinsch and Staskawicz, 1996), *avrB* (Huynh et al., 1989), and *avrPphB* (Puri et al., 1997).

Bacterial strains were inoculated from a single colony into King's Broth (KB) media (Sigma–Aldrich, MO) with appropriate antibiotics and grown at 28 °C overnight. Overnight cultures were diluted into fresh KB media and grown until bacteria were in log

phase. Cultures were resuspended in 10 mM $MgSO_4$ at a dose of approximately 1×10^3 CFU per cm^2 leaf area. For each assay, approximately 0.1 mL of a bacterial suspension was infiltrated into the abaxial sides of 6th–8th true leaves of well-watered four-week-old Arabidopsis plants using a 1 mL syringe without a needle. Following infiltration, flats were covered with clear plastic domes for the duration of the experiment. At indicated time points, a 6 mm diameter cork borer was used to excise leaf punches from infected leaves. Leaf disks were added to a 2.0 mL microcentrifuge tube containing a single 5 mm metal bead and 100 μL of H_2O. The tissue was then ground by subjecting the tubes to 2 min of 30 Hz in a Tissuelyzer II (Qiagen). Ground samples were serially diluted and plated on LB agar plates and grown at 28 °C until colonies were countable (48 h for most strains).

4.4. Callose assay

Leaves of four-week-old plants were infiltrated with bacteria as described above. Heat-killed bacterial suspensions were prepared by submerging diluted bacteria at the indicated titers just prior to infiltration in a 100 °C water bath for 10 min. Eighteen hours after infiltration, leaves were removed and vacuum infiltrated with 3:1 ethanol:acetic acid. The clearing solution was replaced periodically until the leaves became colorless. The leaves were washed in 70% and 50% ethanol sequentially for at least 2 h each, before being rehydrated in several brief H_2O washes followed by an overnight H_2O wash. Samples were then further cleared for several minutes by vacuum infiltration in 10% NaOH followed by a 2 h incubation at 37 °C on a shaking platform. After several more H_2O washes, leaves were incubated in the dark at room temperature for at least 4 h with 0.01% aniline blue in 150 mM K_2HPO_4 (pH 9.5). After mounting on slides in 50% glycerol, samples were examined using a Zeiss Axioplan microscope (Oberkochen, Germany) utilizing UV illumination and a broadband DAPI filter set (excitation filter 390 nm; dichroic mirror 420 nm; emission filter 460 nm). At least 6 leaves from 3 different plants were examined for each treatment and the amount of callose deposition in representative leaves is shown in the figures.

4.5. Hypersensitive response assay

Cell death was visualized by infiltrating leaves with appropriate strains of *P. syringae* pv. *tomato* strain DC3000 (*Pto*) expressing various *avr* genes at a dose of approximately 1×10^3 CFU per cm^2 leaf area as described above and allowing the infection to proceed for 24 h. At that time, infected leaves were excised and submerged in trypan blue stain solution (2.5 mg/mL trypan blue, 25% w/v lactic acid, 23% H_2O saturated phenol, 25% glycerol) pre-heated to 70 °C. Submerged samples were subjected to two periods of vacuum infiltration of 5 min each in the trypan blue stain solution and then heated by steam above a boiling water bath for 2 min. Once cooled, the staining solution was replaced by successive washes of chloral hydrate (15 g/10 mL H_2O) until the leaves were cleared. Several H_2O washes were followed by an overnight incubation in H_2O and several hours equilibration in 70% glycerol before mounting for photography. Ion leakage was measured by infiltrating leaves with *Pto* DC3000 (*avrRpm1*) at 5×10^7 CFU/mL as described above. Four hours after infiltration, leaf disks were removed using a 6 mm cork borer and floated in 500 μL diH_2O in a 12-well plate, abaxial side down, for 3 h. Ion conductivity was measured using a Radiometer Copenhagen CDM3 Conductivity Meter.

4.6. RNA isolation and quantitative RT-PCR

RNA was isolated according to the manufacturer's instructions using the RNeasy Plant Mini Kit (Qiagen). DNA was removed using the DNA-free kit (Ambion) and the RT reaction was done using the iScript cDNA synthesis kit (Bio-rad). cDNA concentrations were measured on a Nano-drop (Thermo Scientific). The following PCR reaction program was used: 95 °C for 3 min following by 50 cycles of 95 °C for 30 s followed by 55 °C for 30 s. Fold-change was calculated using the Pfaffl method (Pfaffl, 2001). The primers used were: *EIF4A* (5′-GCAGTCTCTTCGTGCTGACA-3′ and 5′-TGTCATAGATC TGGTCCTTGAA-3′); *PR1* (5′-ACACCTCACTTTGGCACATC-3′ and 5′-GAGTGTGGAAAACGCAAAGA-3′); *GST6* (5′-CCATCTTCAAAGGCTG-GAAC-3′ and 5′-TCGAGCTCAAAGATGGTGAA-3′); *PR2* (5′-CCTTCT CGGTGATCCATTCT-3′ and 5′-AGTGTGGAAAACGCAAAGACT-3′); *PDF1.2* (5′-TCACCCTTATCTTCGCTGCT-3′ and 5′-TCGCACAACTTCTG TGCTTC-3′); *VSP1* (5′-CTCAAGCCAAACGGATCG-3′ and 5′-TTCCCAA CGATGTTGTACCC-3′); *PR3* (5′-GTATGGCTGGACCGCCTTC-3′ and 5′-GTTCTTCACCCTTAAACACTTGC-3′); *PR4* (5′-GTTGCTGCATTGGT CCACTA-3′ and 5′-GGGTGAAGAACACAAGAACAAA-3′); *ERS1* (5′-AG TTCCACGGTCTGGTTTGT-3′ and 5′-GTAAACGGTTTGTCGGGCTA-3′); *MYB51* (5′-CTTGTGTGTAACTGGATCAA-3′ and 5′-ACAAATGGTCTG CTATAGCT-3′).

4.7. nanoString transcriptomic analysis

A panel of SA-related genes (see Table 1) was chosen to create a nanoString barcode set (Geiss et al., 2008). Leaves of four-week old Arabidopsis Col-0 plants were excised before or 48 h after infiltration with *Pto* DC3000 or *Pto* DC3000 expressing *avrRpm1*. RNA was isolated from infected leaves and the relative levels of the selected mRNAs were measured according to the manufacturer's instructions using the relevant custom synthesized barcodes, a nanoString Preparation Station, and an nCounter Digital Analyzer (nanoString Technologies).

4.8. Detection of NPR1 protein by immunoblotting

Seeds of Arabidopsis ecotype Col-0 wild type and *asFBP1.1* transgenic lines were sown on soil (Metro Mix 200; Grace-Sierra, Milpitas, CA) and kept at 4 °C in a cold room for 3 days before being moved into a growth room at 22 °C under a 16 h light and 8 h dark cycle. Two weeks later, the seedlings were transferred into individual pots. Four-week-old plants were sprayed with 0.5 mM salicylic acid, and leaves were harvested at 0, 4, 8 and 24 h after the treatments. Total protein was isolated by homogenizing the leaf tissue in a protein extraction buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.1% Triton X-100, 0.2% Nonidet P-40, and inhibitors: 50 μg/mL TPCK, 50 μg/mL TLCK, 0.6 mM PMSF, 40 μM MG115) (Fan and Dong, 2002). The homogenates were cleared of cell debris by centrifugation twice at 12,000 rpm for 10 min each. Supernatant protein was denatured in the SDS sample buffer with (reducing condition) and without (non-reducing condition) 100 mM DTT at 75 °C for 10 min. The total protein was then separated on a Novex® 12% precast Tris–Glycine gel (Life Technology, Carlsbad, CA), transferred onto a nitrocellulose membrane, and immunoblotted using an NPR1 antibody (Mou et al., 2003).

4.9. Statistical analysis

Student's *t* test was performed to calculate whether the differences between distributions of data were significant using PRISM v4.0 (GraphPad Software). A p value of <0.05 was considered statistically significant.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2014.07.010.

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