Journal of General Plant Pathology (2019) 85:405–412
https://doi.org/10.1007/s10327-019-00863-9

HOST RESPONSES

A class III peroxidase PRX34 is a component of disease resistance in Arabidopsis

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Received: 2 January 2019 / Accepted: 12 February 2019 / Published online: 7 June 2019
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Abstract

PRX34 mediates the oxidative burst in Arabidopsis. Here we characterized two additional Arabidopsis *prx34* null mutants (*prx34-2*, *prx34-3*), besides the well-studied *prx34-1*. Due to a decrease in corresponding peroxidase, the activity that generates reactive oxygen species (ROS) was significantly lower in cell wall extracts of *prx34-2* and *prx34-3* plants. Consistently, the *prx34-2* and *prx34-3* exhibited reduced accumulation both of ROS and callose in Flg22-elicitor-treated leaves, leading to enhanced susceptibility to bacterial and fungal pathogens. In contrast, ectopic expression of *PRX34* in the wild type caused enhanced resistance. PRX34 is thus a component for disease resistance in Arabidopsis.

Keywords Apoplastic oxidative burst · Arabidopsis · Cell wall · Class III peroxidase · PRX34 · Reactive oxygen species (ROS)

Introduction

Apoplastic class III peroxidases (EC 1.11.1.7) comprise a large protein family thought to be involved in diverse functions in plants due to their catalytic versatility and numerous isoforms (Shigeto and Tsutsumi 2016; Tognolli et al. 2002; Welinder et al. 2002). Peroxidases usually catalyze the oxidation of substrates via hydrogen peroxide (H_2O_2), whereas some isoforms of this family can generate reactive oxygen species (ROS) at a neutral to basic pH in the presence of some reductants (Bolwell et al. 1998, 2002; Kawano 2003; Kimura and Kawano 2015; O'Brien et al. 2012a). To date, the apoplastic peroxidase-dependent oxidative burst in response to an elicitor or microbe-associated molecular pattern (MAMP) has been validated in French bean (Bolwell et al. 1998), Arabidopsis (Bindschedler et al. 2006; Davies et al. 2006; O'Brien et al. 2012b), pepper (Choi et al. 2007), lettuce (Bestwick et al. 1998) and pea (Kiba et al. 1997).

The Arabidopsis genome encodes 73 class III peroxidases (*PRX1* through *PRX73*), among which subsets of the family are upregulated by pathogens, salicylic acid (SA), jasmonic acid and ethylene (Almagro et al. 2009), but the precise role is still poorly understood for each isoform, due to the great complexity of genetic analysis (Oliva et al. 2009; Welinder et al. 2002). Under such circumstances, Bindschedler et al. (2006) constructed transgenic Arabidopsis plants expressing an antisense cDNA encoding a French bean class III peroxidase (FBP1) to identify key peroxidases involved in ROS generation in Arabidopsis. The antisense expression of a heterologous FBP1 cDNA in *Arabidopsis thaliana* resulted in diminished expression of at least two genes, *PRX33* (*At3g49110*) and *PRX34* (*At3g49120*), and in an attenuated oxidative burst in response to a fungal elicitor, leading to

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10327-019-00863-9) contains supplementary material, which is available to authorized users.

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enhanced susceptibility to a range of fungal and bacterial pathogens (Bindschedler et al. 2006). Further investigation of this knockdown line as well as the *prx33-1* and *prx34-1* T-DNA insertion mutants demonstrated that all of these lines are partially impaired in pattern-triggered immunity (PTI), resulting in enhanced susceptibility to fungal and bacterial pathogens (Bindschedler et al. 2006; Daudi et al. 2012). However, the *prx34-1* mutant (ecotype Columbia-0 [Col-0]) containing the T-DNA insertion in the promotor region of *PRX34* gene is likely a hypomorphic mutant (Arnaud et al. 2017; Passardi et al. 2006). Actually, we detected transcripts from mature leaves of this mutant using PCR with reverse transcription (RT-PCR) (Fig. 1b), suggesting that the expression of *PRX34* was not severely abolished. In addition, the tandem-duplicated *PRX33* gene in the *prx34-1* mutant was unwantedly affected under our laboratory conditions. Although PTI responses such as ROS generation and callose accumulation were impaired in the mutant, probably due to a decrease in *PRX33* transcripts, the *prx34-1* mutant did not have the enhanced susceptibility phenotype when challenged with virulent pathogens such as *Botrytis cinerea*, *Colletotrichum higginsianum* and *Pectobacterium carotovorum* subsp. *carotovorum* (Fig. S1). Accordingly, different allelic defects are needed to unambiguously confirm the function of *PRX34* in disease resistance. In this study, we obtained two additional T-DNA insertion null mutants (referred to as *prx34-2* and *prx34-3*) and characterized these mutants for their responses to bacterial and fungal pathogens. We also generated transgenic Arabidopsis plants that overexpress *PRX34* to unravel the function of the corresponding peroxidase in disease resistance.

Arabidopsis thaliana ecotype Col-0 was used as the wild type. The *prx34-1* (SALK_051769) and *prx34-2* (GK-728F08) mutants were purchased from the Salk T-DNA insertion line (Alonso et al. 2003) and the Nottingham Arabidopsis Stock Centre, respectively. Homozygous T-DNA insertion mutants in *PRX34* (*prx34-1* and *prx34-2*) were selected by PCR with loci-specific primers in combination with T-DNA border primer (BP) (Fig. 1a; Fig. S2). A homozygous *prx34-3* (SALK_112466C) was also obtained from the Salk T-DNA insertion line (Fig. 1a; Fig. S2). The absence of transcripts was confirmed by quantitative RT-PCR. Seeds were sown in a 1:1 mixture of vermiculite–peat (Supermix-A soil; Sakata Seed Co., Ltd., Yokohama, Japan) and grown in a chamber at 22 °C, with a 10 h light/14 h dark cycle at 11.8 $W \cdot m^{-2}$. For the overexpression experiment, we amplified the open reading frame of the *PRX34* by RT-PCR with complementary DNA from Arabidopsis seedlings, cloned it downstream of the CaMV 35S promoter in pENTR4m (Matsui et al. 2017) using an In-Fusion HD Cloning Kit (Clontech, Palo Alto, CA, USA), and subsequently recloned it into a binary vector pGWB2 (Nakagawa et al. 2007) using LR clonase II enzyme mix (Thermo Fisher Scientific, Waltham, MA, USA). Two independent transgenic Arabidopsis plants were generated by the standard floral dip method (Clough and Bent 1998).

First, we characterized three available Arabidopsis lines homozygous for T-DNA insertions (Fig. 1a). Similar to previous observations (Daudi et al. 2012; Lyons et al. 2015; Passardi et al. 2006), *PRX34* expression was not completely abolished in the *prx34-1* mutant (Fig. 1b), so it was excluded from further analyses. In contrast, no transcripts were detected in either *prx34-2* or *prx34-3* mutants by quantitative RT-PCR (Fig. 1b), suggesting loss-of-function mutations. Obviously, *PRX33* expression was not affected in these mutants. In parallel, we also obtained NaCl-solubilized cell wall proteins from individual plants as described previously (Kiba et al. 1997; Toyoda et al. 2012) and analyzed accumulation of the corresponding peroxidase with an anti-horseradish peroxidase (HRP) antibody (Fig. 1c). In contrast to considerable accumulation in the wild type, protein accumulation was negligible or not detectable in mutants *prx34-2* and *prx34-3*, but not in *prx34-1* (Fig. 1c). Together with the analysis of transcripts (Fig. 1b), this result indicates that PRX34 is one of the major peroxidases that constitutively accumulate in the mature leaves of Arabidopsis plants. The *prx34-2* and *prx34-3* mutants grew similar to the wild type (Fig. 1d).

To examine the role of PRX34 in ROS generation, we tested NaCl-solubilized cell wall proteins from the wild type and mutants *prx34-2* and *prx34-3* for their ROS-generating activity. Remarkably, the ROS generation that requires manganese ion (Mn^{2+}), *p*-coumaric acid (*p*-CA) and NADH as an electron donor was significantly lower in the extracts from mutants *prx34-2* and *prx34-3*, likely due to a decrease in the corresponding peroxidases (Fig. 1e). Consistent with this result, salicylhydroxamic acid (SHAM) significantly reduced ROS levels, suggesting that PRX34 is closely associated with ROS generation in the apoplast (Fig. 1e). Gross et al. (1977) and Halliwell (1978) reported, using cell-wall-bound peroxidases or purified horseradish peroxidases, the oxidation of NADH leads to the generation of O_2^-/H_2O_2 through a complex pathway involving apoplastic NADH, NAD and NAD^+. Halliwell (1978) also demonstrated that peroxidase-catalyzed H_2O_2/O_2^- generation largely depends on both Mn^{2+} and phenolic compounds such as *p*-CA. In fact, exclusion of either NADH, Mn^{2+} or *p*-CA significantly lowered the O_2^--generating activity in extracts from Arabidopsis cell walls (data not shown), confirming that cell wall peroxidase(s) itself is capable of generating ROS through the oxygen-requiring cycle independently of H_2O_2 as reported previously (Kimura and Kawano 2015). Collectively, PRX34 is likely one of the important isoforms that generate ROS through the oxidation of NADH.

Next, we further investigated whether PRX34 contributes to disease resistance in Arabidopsis by inoculating *prx34*

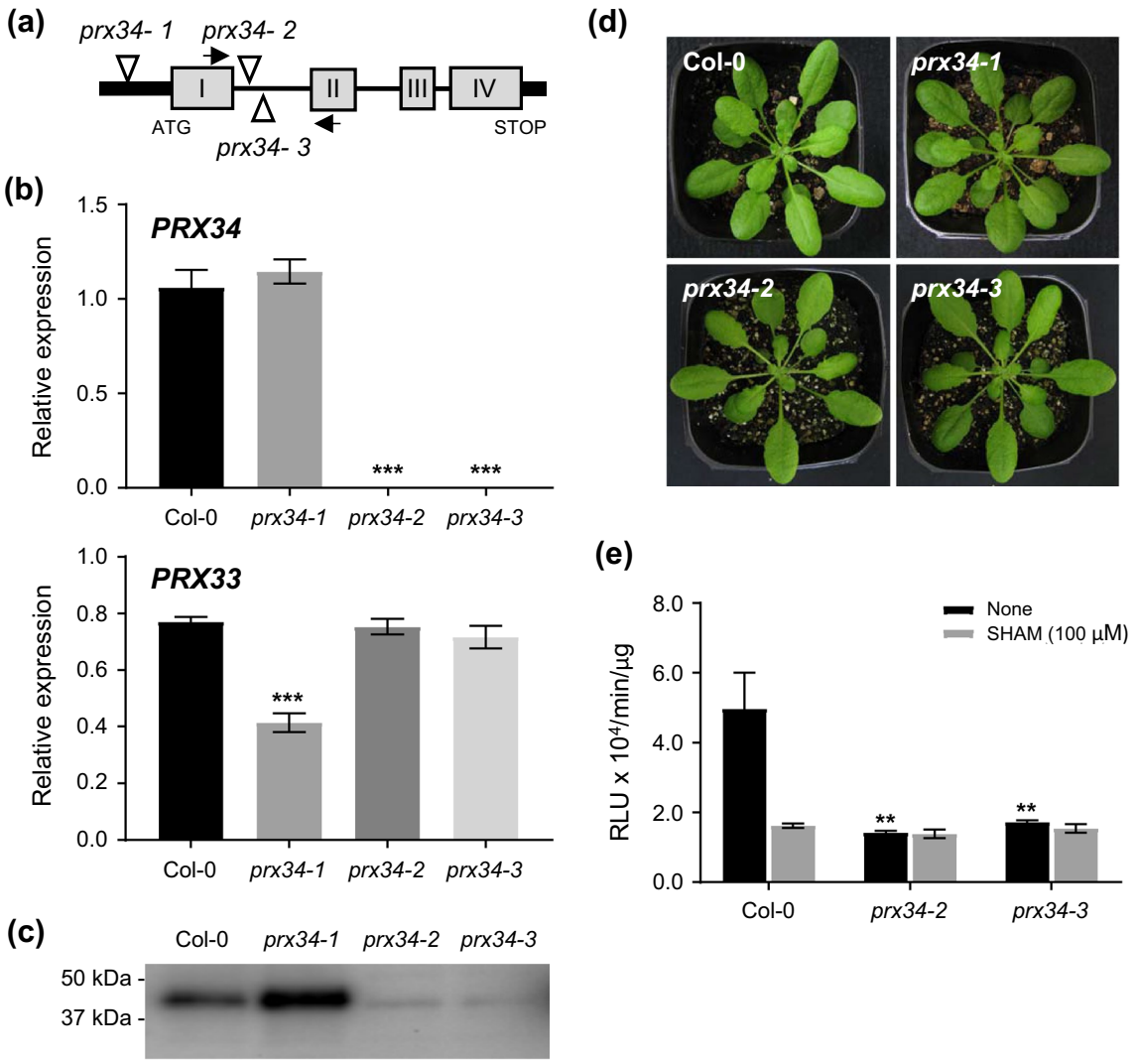

Fig. 1 Phenotypic and molecular characterization of *atprx34* mutants. **a** Gene organization of *AtPRX34* (*At3g49120*) and insertion sites of T-DNA. Grey boxes represent exons (numbered from I to IV) and black lines introns. Bold lines are the predicted 5′ and 3′ UTRs. Arrows indicate primer set used for transcript analysis. **b** *PRX34* transcript levels in 4 week-old seedlings of wild type and mutants *prx34-1*, *prx34-2* and *prx34-3*. Quantitative RT-PCR was performed with a Shimadzu GVP-9600 Gene Detection System (Shimadzu, Kyoto, Japan), using primer sets listed in Table S2. The expression level of each gene was normalized using *EF1-α* (*At5g60390*). *PRX33* was quantified similarly. Data are shown as the average ± standard deviation (SD) from three independent plants. Asterisks indicate significant difference (Dunnett's test; ***$p < 0.001$). **c** Western blot analysis of peroxidase accumulation in 4 week-old seedlings of the wild type and *prx34* mutants. NaCl-solubilized cell wall proteins were obtained as described previously (Kiba et al. 1997; Toyoda et al. 2012). Equal amounts of cell wall proteins (1.5 μg) were separated by SDS–PAGE and transferred onto a PVDF membrane, probed with anti-horseradish peroxidase (HRP) antibody (1:10,000; GTX22110; GeneTex, Irvine, CA, USA). **d** Four-week-old seedlings. **e** Generation of ROS in cell wall extracts though NADH oxidation. Cell wall proteins (0.25 μg) from individual plants were incubated in a reaction mixture (50 μl) containing 30 mM Tris/MES (pH 6.5), 0.5 mM NADH, 0.5 mM *p*-coumaric acid (*p*-CA) and 20 mM $MnCl_2$ and 10 μM MPEC (ATTO Co., Ltd., Tokyo, Japan) without or with 100 μM of salicylhydroxamic acid (SHAM). Chemiluminescence (RLU) was measured with a Lumat LB9507 (Berthold Technologies, Bad Wildbad, Germany) for 2 min. Data represent the average ± SD of three replicates. Asterisks indicate significant difference (Dunnett's test; ***$p < 0.001$)

mutants with virulent strains of fungal and bacterial pathogens such as *Botrytis cinerea*, *Colletotrichum higginsianum* and *Pectobacterium carotovorum* subsp. *carotovorum*. Interestingly, regardless of the pathogen tested, the drop inoculation assays clearly showed that, compared to the wild type, mutants *prx34-2* and *prx34-3* developed significantly larger lesions (Fig. 2a). Consistent with this result, these mutants had reduced PTI responses such as accumulation both of ROS and callose when exposed to Flg22 peptide as a bacterial MAMP (Fig. 2b, c). These results indicate that PRX34 is a component of PTI and that it is necessary for full resistance to virulent pathogens.

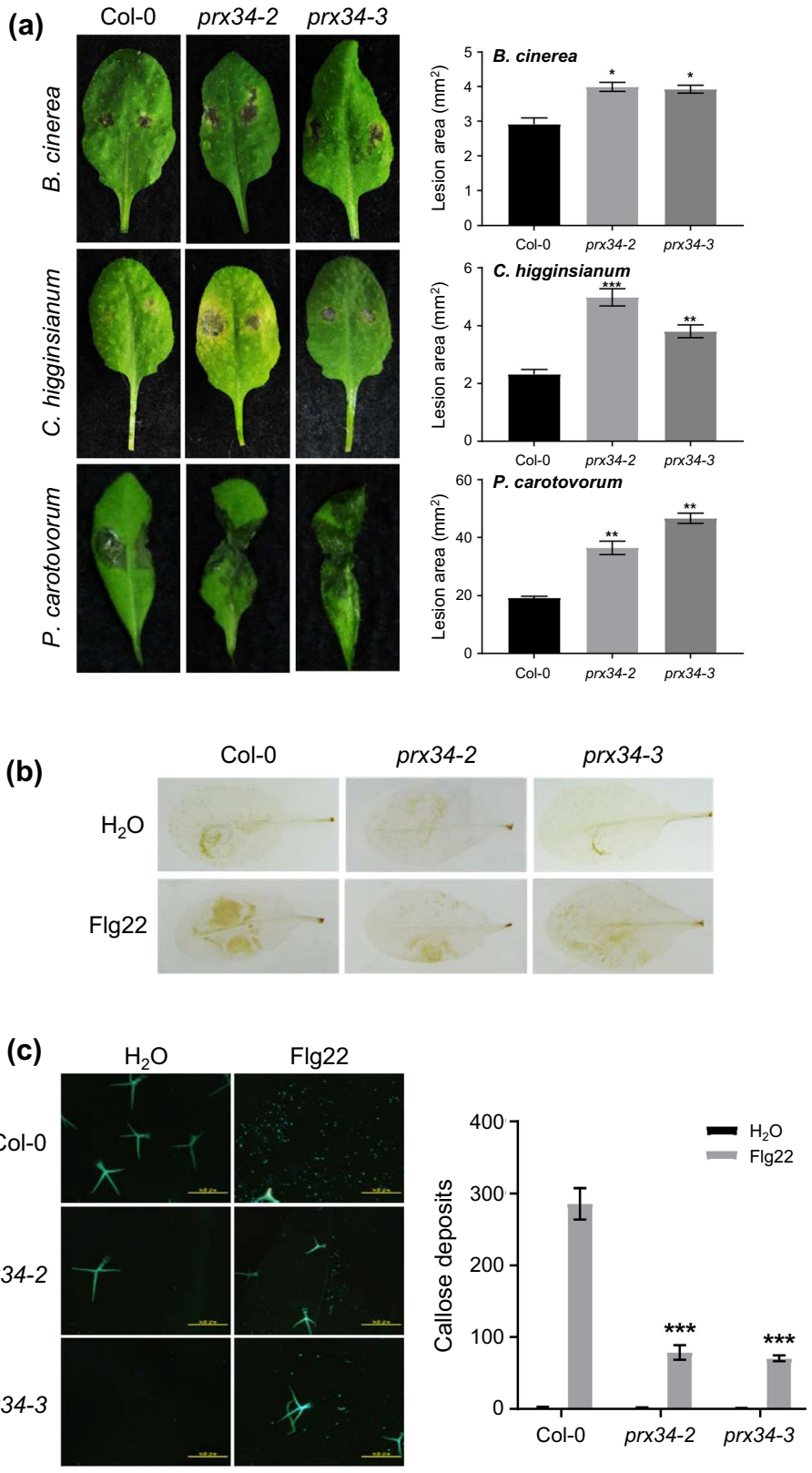

Fig. 2 Enhanced susceptibility of *prx34* mutants to fungal and bacterial pathogens. **a** Symptoms induced by *Botrytis cinerea* (MAFF712189), *Colletotrichum higginsianum* (MAFF305635) and *Pectobacterium carotovorum* subsp. *carotovorum* strain Pc1. Inoculations: *B. cinerea* and *C. higginsianum*, 5-µl drop of 2×10^5 conidia/ml in distilled water or 1% Sabouraud maltose broth was placed on each side of the mid vein of detached leaves of 4 week-old wild type, *prx34-2* and *prx34-3*; *P. carotovorum* subsp. *carotovorum* strain Pc1, 5 µl drop of 1×10^5 cfu/ml was placed on wounded leaves. All inoculated leaves were incubated for 2 or 3 days at 22 °C, then photographed, and lesions were measured. Data are the average ± SD of 10 leaves from 5 independent plants. Asterisks indicate significant difference (Dunnett's test: $*p<0.05$; $**p<0.01$; $***p<0.001$). **b** Flg22-elicited ROS generation in Arabidopsis leaves. Mature leaves of 4 week-old seedlings of the wild-type and mutants *prx34-2* and *prx34-3* were infiltrated with approximately 0.1 ml of 100 nM Flg22 or water (as control). Leaves were stained with 3,3′-diaminobenzidine (DAB) at 2 h after treatment to detect the accumulation of H_2O_2. Experiments were repeated three times with similar results. **c** Flg22-elicited callose accumulation detected by aniline blue staining 24 h after treatment. Mature leaves were infiltrated with approximately 0.1 ml of 100 nM Flg22 or water (as control) as described above. The number of callose deposits was calculated using ImageJ software. Data are the average ± SD of 10 leaves from five independent plants Asterisks indicate significant difference (Dunnett's test; $***p<0.001$)

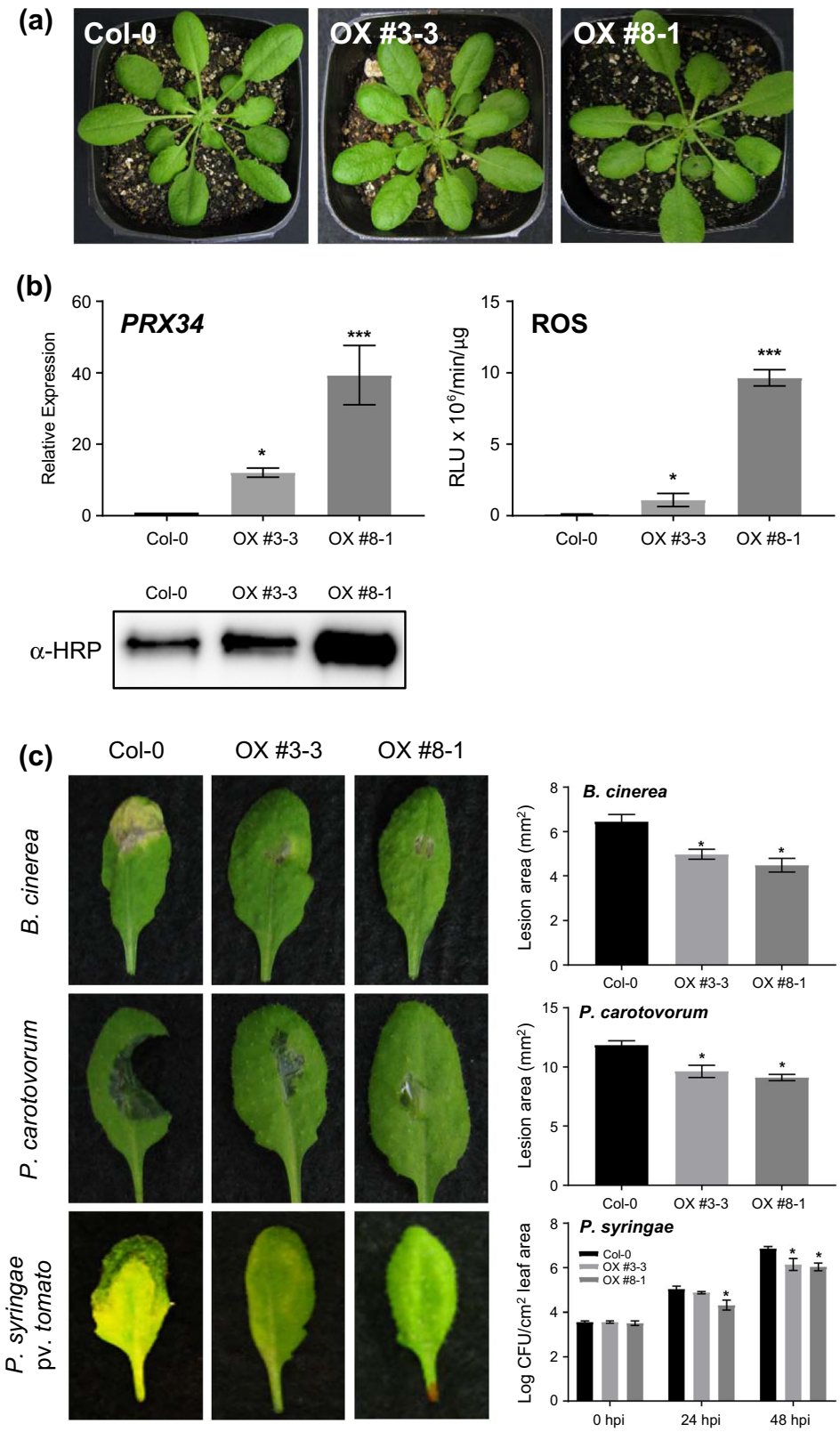

Fig. 3 Enhanced resistance of *PRX34*-overexpressors OX #3-3 and OX #8-1 to fungal and bacterial pathogens. **a** Growth of 4 week-old seedlings of OX #3-3 and OX #8-1. **b** *PRX34* transcript levels in mature leaves of 4 week-old seedlings of wild type, OX#3-3 and OX #8-1 (left). Inset shows accumulation of peroxidase proteins probed with anti-HRP antibody. NaCl-solubilzed cell-wall extracts of wild type, OX#3-3 and OX#8-1 were also tested for ROS-generating activity as described in Fig. 1e legend (right figure). **c** Overexpression of *PRX34* caused enhanced resistance to virulent strains of fungal and bacterial pathogens. Inoculation with *Botrytis cinerea* and *Pectobacterium carotovorum* subsp. *carotovorum* is described in Fig. 2a legend. All inoculated leaves were incubated for 2 or 3 days at 22 °C, then photographed, and lesions were measured. For inoculation with *Pseudomonas syringae* pv. *tomato* DC3000, leaves of wild type, OX #3-3 and OX #8-1 were infiltrated with a bacterial suspension (1×10^7 cfu/ml) and bacteria cells were counted 1 and 2 days after inoculation. All inoculated leaves were incubated similarly, then photographed 3 days after inoculation. Data are mean bacterial titer ± SD of eight leaf disks excised from 8 leaves of 4 independent plants. Asterisks indicate a significant difference (Dunnett's test; $^{**}p < 0.05$)

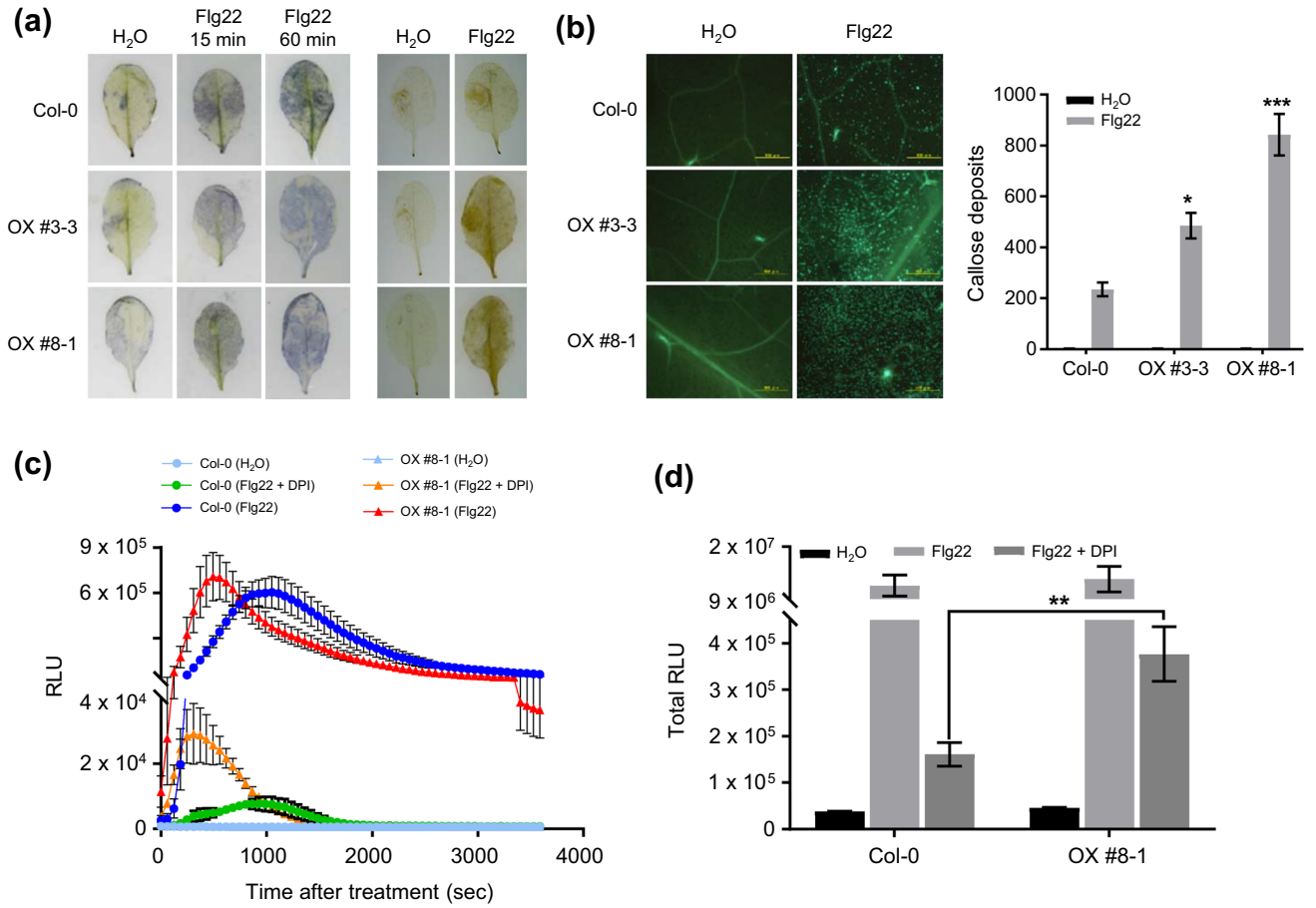

Fig. 4 Phenotypic and molecular characterization of Arabidopsis plants expressing *PRX34*. **a** NBT and DAB staining to detect superoxide (O_2^-) and H_2O_2, respectively. Leaves of 4-week-old plants of the wild type, OX#3-3 and OX #8-1 were treated with 100 nM Flg22 or water (as control). Respective staining was performed for 15 or 60 min after treatment. For DAB staining, the leaves were stained 2 h after treatment. Experiments were repeated at three times with similar results. **b** Callose accumulation in Flg22-treated leaves of the wild type and *PRX34*-overexpressors OX #3-3 and OX #8-1. Leaves were treated with 100 nM of Flg22 or water (as control) for 24 h similarly and then stained with aniline blue to detect callose accumulation. The number of callose deposits was calculated using ImageJ software. Data represent the average ± SD of 10 leaves from five independent plants. Asterisks indicate significant difference (Dunnett's test; $*p < 0.05$; $***p < 0.001$). **c** Kinetic analysis of ROS generated in the wild type and OX #8-1 in the absence or presence of diphenylene iodonium (DPI), a chemical inhibitor of NADPH oxidase. Leaf disks were exposed to 100 nM of Flg22 in a solution containing 200 µM L-012 (Fujifilm Wako Pure Chemical Co. Ltd., Osaka, Japan), in the absence or presence of DPI. ROS generated in the test solution was measured successively with TriStar^2 LB942 (Berthold Technologies, Bad Wildbad, Germany) for 60 min. Data are means ± SD from three biological replicates. **d** Statistical analysis of integrated values of relative light value (RLU) over 3600 s after each treatment described in Fig. 4c. Asterisks indicate significant difference (Dunnett's test; $*p < 0.05$; $***p < 0.001$)

To further define the function of PRX34, we generated two independent lines of Arabidopsis plants expressing the *PRX34* under the control of the CaMV 35S promoter. The overexpression lines, named OX #3-3 and OX #8-1, exhibited normal growth similar to the wild type (Fig. 3a). Western blot analysis with NaCl-solubilized extracts recovered from the wild-type, OX #3-3 and OX #8-1 plants revealed higher levels of the corresponding peroxidase, consistent with the transcript analysis (Fig. 3b). Actually, the activity that generates ROS in the cell wall extracts of the OX #3-3 and OX #8-1 was higher than in the wild type (Fig. 3b). Interestingly, lesion development was restricted in Arabidopsis plants expressing *PRX34* compared to those on the wild type after inoculation with virulent strains such as *B. cinerea*, *P. carotovorum* subsp. *carotovorum* and *Pseudomonas syringae* pv. *tomato* (Fig. 3c). This result is consistent with our finding that increased expression of *PRX34* produced more ROS and callose than in the wild-type plants after infiltration with Flg22 (Fig. 4a, b).

Finally, leaf disks from the wild type and OX #8-1 plants were exposed to 100 nM of Flg22 in a solution containing 200 µM L-012, with or without diphenylene iodonium

(DPI), a chemical inhibitor of NADPH oxidase, to ascertain the role of peroxidase in the early oxidative burst. Although no obvious difference in the Flg22-elicited oxidative burst was observed between the wild type and OX #8-1, the ROS burst started earlier in OX #8-1 (Fig. 4c), suggesting that the increased PRX34 has a role in the early oxidative burst. Indeed, due to increased levels of PRX34, OX #8-1 produced DPI-insensitive ROS, unlike the wild type (Fig. 4c). This result was also confirmed by a statistical analysis of integrated values of relative light value (RLU) for 3600 s after each treatment; the value of the Flg22-elicited OX #8-1 with DPI was constantly higher than in the wild type (Fig. 4d).

In this study, we evaluated the role of Arabidopsis class III PRX34, especially in terms of the Flg22-elicited oxidative burst as an early (~ 10 min) response and callose formation as a late (~ 24 h) response. In Arabidopsis plants lacking *PRX34*, nearly all Flg22-elicited callose was abolished (Fig. 4c). Importantly, both *prx34-2* and *prx34-3* plants had enhanced susceptibility to virulent pathogens, whereas ectopic expression of *PRX34* improved resistance (Figs. 3a, 4a). Mammarella et al. (2015) reported recently that in Arabidopsis, SA-mediated activation of *PR1* was impaired in *PRX34*-silenced plants. Furthermore, the expression of defense-related genes such as *PR1*, *WRKY38* and *WRKY54* after infection with a virulent strain of *P. syringae* pv. *tomato* was clearly diminished when *PRX34* was silenced. Taken together, our results suggest that a certain isoform(s) such as PRX34 substantially contributes to both early and late responses during PTI. Indeed, *PRX34* is upregulated after infection or in response to an elicitor such as Flg22 and chitin (Bindschedler et al. 2006; Daudi et al. 2012; Fig. S3). Further experiments are needed to explore the distinctive roles of plant class III peroxidase(s) in the early oxidative burst and the late responses including downstream defense-signaling pathway during PTI.

Acknowledgements We acknowledge Prof. Dr. Shinji Tsuyumu (Laboratory of Plant Pathology, Faculty of Agriculture, Shizuoka University, Shizuoka, Japan) for generously providing *Pectobacterium carotovorum* subsp. *carotovorum* strain Pc1. This research was supported in part by the Grants-in-Aid for Scientific Research (18K05645) from the Japan Society for Promotion of Science (JSPS).

Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflict of interest.

Research involving human and/or animal participants This article does not contain any studies with human participants or animals performed by any of the authors.

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