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ORIGINAL ARTICLE

Enhanced disease resistance in transgenic carrot (*Daucus carota* L.) plants over-expressing a rice cationic peroxidase

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Abstract Plant class III peroxidases are involved in numerous responses related to pathogen resistance including controlling hydrogen peroxide (H_2O_2) levels and lignin formation. Peroxidases catalyze the oxidation of organic compounds using H_2O_2 as an oxidant. We examined the mechanisms of disease resistance in a transgenic carrot line (P23) which constitutively over-expresses the rice cationic peroxidase *OsPrx114* (previously known as *PO-C1*) and which exhibits enhanced resistance to necrotrophic foliar pathogens. *OsPrx114* over-expression led to a slight enhancement of constitutive transcript levels of pathogenesis-related (*PR*) genes. These transcript levels were dramatically increased in line P23 compared to controls [GUS construct under the control of 35S promoter (*35S::GUS*)] when tissues were treated with cell wall fragments of the fungal pathogen *Sclerotinia sclerotiorum* (SS-walls), and to a lesser extent with 2,6-dichloroisonicotinic acid. There was no basal increase in basal H_2O_2 levels in tissues of the line P23. However, during an oxidative burst response elicited by SS-walls, H_2O_2 accumulation was reduced in line P23 despite, typical media alkalinization associated with oxidative burst responses was observed, suggesting that OsPrx114 was involved in rapid H_2O_2 consumption during the oxidative burst response. Tap roots of line P23 had increased lignin formation in the outer periderm tissues, which was further increased during challenge inoculation with *Alternaria radicina*. Plant susceptibility to a biotrophic pathogen, *Erysiphe heraclei*, was not affected. Disease resistance to necrotrophic pathogens in carrot as a result of *OsPrx114* over-expression is manifested through increased PR transcript accumulation, rapid removal of H_2O_2 during oxidative burst response and enhanced lignin formation.

Keywords Peroxidase · Lignin · Hydrogen peroxide · Pathogen resistance · Oxidative burst

Abbreviations

PR	Pathogenesis related
Prx	Peroxidase
GUS	β-Glucuronidase
JA	Methyl-jasmonic acid
INA	2,6-Dichloroisonicotinic acid
SS-walls	*Sclerotinia sclerotiorum* cell wall fragments
DAB	3′,3-Diaminobenzidine
AIR	Alcohol-insoluble residue
LTGA	Ligno-thioglycolic acid derivative

Introduction

Plant class III peroxidases (Prx) are present in all higher plants and are encoded by a large gene family which can comprise of over 70 genes (Passardi et al. 2005). A large number of peroxidase genes are induced during pathogen challenge. For example, up to ten peroxidase genes were upregulated in *Magnaporthe*-infected rice (Sasaki et al. 2004). In Arabidopsis plants, seven and three peroxidase genes were upregulated when challenged with *Pseudomonas* spp. (Mohr and Cahill 2007) and *Botrytis cinerea*

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(Chassot et al. 2007), respectively. Plant peroxidases catalyze the oxidation of a range of organic substrates using hydrogen peroxide (H_2O_2) as an oxidant (Hiraga et al. 2001). As well, many different biochemical functions in plant pathogen defense responses are associated with peroxidases. These include lignin formation (El Mansouri et al. 1999; Lagrimini 1991; Quiroga et al. 2000), xylem wall thickening (Hilaire et al. 2001) and phytoalexin biosynthesis (Kristensen et al. 1999). Peroxidases have also been shown to be involved in the generation of reactive oxygen species (Bestwick et al. 1997; Bindschedler et al. 2006) and scavenging of H_2O_2 (Baker et al. 2000; Kawaoka et al. 2003). These reports indicate that peroxidases have multiple functions in plant defenses against both biotic and abiotic stresses (Passardi et al. 2005). Since the functions of peroxidases are broad and the physiological reactions they are involved with are complex, it has been difficult to definitely demonstrate the role of peroxidases in plant–pathogen interactions.

In previous reports, a reduction of peroxidase activity in Arabidopsis (Bindschedler et al. 2006) and bell pepper plants (Choi et al. 2007) resulted in reduced H_2O_2 accumulation and enhanced susceptibility to biotrophic fungal and bacterial pathogens. Similarly, over-expression of peroxidase resulted in increased H_2O_2 accumulation and enhanced tolerance to biotrophic pathogens in Arabidopsis (Choi et al. 2007), wheat (Schweizer 2008) and tobacco plants (Kim et al. 2008). In contrast, antisense suppression of a tomato peroxidase *LePrx06* (previously *Ep5C*) resulted in increased H_2O_2 accumulation and enhanced resistance to *Pseudomonas syringe* pv. *tomato* (Coego et al. 2005), indicating that LePrx06 may be involved in reduction of H_2O_2 levels. These reports indicate that different peroxidases function in distinct manners, with certain peroxidases involved in defense against biotrophic pathogens while others may function in necrotrophic interactions.

We previously generated transgenic carrot plants that constitutively over-express the rice *OsPrx114* (previously referred to as *PO-C1*) gene (Wally et al. 2009a), which encodes for a pathogen-inducible cationic class III (PR-9) peroxidase. In rice, this gene was induced to high levels following inoculation with avirulent strains of *Xanthomonas oryzae* (Young et al. 1995). *OsPrx114* encodes a 311 amino acid peptide with a putative N-terminus extracellular localization signal. Extracellular transport was confirmed by immune-localization of inoculated rice (Hilaire et al. 2001) as well as enhanced cell wall bound enzyme fractions in transgenic carrots (Wally et al. 2009a). Currently, there are only short carrot peroxidase protein sequences of 10–17 amino acids which have only limited similarity. The highest degree of amino acid similarity to OsPRx114 found in wheat peroxidase TaPrx103 (Altpeter et al. 2005; Schweizer 2008) and barley HvPrx08 (Johrde and Schweizer 2008). In the transgenic plant transformed with the construct *ubi::OsPrx114* referred to as line P23, peroxidase enzyme activity was increased by 3.5-fold in the cell wall ionically bound protein fraction (Wally et al. 2009a). The *OsPrx114* gene plays an important role in the early defense responses in resistant rice cultivars and was proposed to be a putative lignin-forming enzyme (Hilaire et al. 2001). In our previous work, the *OsPrx114* expressing carrot lines were highly resistant to the foliar necrotrophic pathogens *Sclerotinia sclerotiorum* and *Botrytis cinerea,* without showing any visible phenotypic abnormalities (Wally et al. 2009a). This was the first report of peroxidase over-expression in plants which has resulted in disease resistance against necrotrophic pathogens.

In the present study, we explored the possible mechanisms by which *OsPrx114* expression could enhance resistance to necrotrophic fungal pathogens in root and foliar tissues by quantifying pathogenesis-related (PR) protein gene expression, as well as H_2O_2, lignin and phenolic production, in transgenic line compared to control carrot lines transformed with *35S::GUS* construct.

Materials and methods

Transgenic carrot plants

Primary transformants of transgenic 'Nantes Coreless' carrots constitutively over-expressing the rice cationic peroxidase gene *OsPrx114* under the control of the maize ubiquitin promoter, or *35S::GUS* control plants containing the *35S::GUS* chimera construct developed in previous studies (Wally et al. 2008, 2009a), were grown under greenhouse conditions or maintained in suspension cultures in MS media supplemented with 0.5 mg l^{-1} 2,4-dichlorophenoxyacetic acid (Chen and Punja 2002). Several lines expressing OsPrx114 were resistant to *B. cinerea* and *S. sclerotiorum,* line P23 was selected for analysis since this line exhibited the highest levels of pathogen resistance (Wally et al. 2009a).

Chemicals

All chemicals were obtained from Sigma (Oakville, ON, Canada) unless otherwise noted.

Effect of OsPrx114 on PR transcript levels

Total RNA was extracted from lyophilized carrot suspension cultures (~250 mg fresh weight) from the *OsPrx114* line P23 and the *35S::GUS* line using the monophasic RNA extraction method (Chomczynski and Sacchi 1987). The suspension cultures were treated with 2,6-dichloroisonicotinic acid (INA) (500 μM), methyl-jasmonic acid (JA)

(100 μM), H_2O_2 (500 μM) or *S. sclerotiorum* cell walls (SS-walls) [100 μg ml^{-1} dry weight (dw)]. The SS-walls were prepared from 7-day-old *S. sclerotiorum* cultures grown on potato dextrose broth at room temperature according to published procedures (Tweddell et al. 1994). The lyophilized SS-walls were suspended in H_2O (10 mg ml^{-1} dw) and sterilized by autoclaving. Ten micrograms of total RNA was separated on a formaldehyde-agarose gel, blotted and bound to Hybond-XL membrane (GE healthcare, Buckinghamshire, UK), and hybridized overnight at 65°C with various ^{32}P labeled probes. Defense genes from carrot including: *phenylalanine ammonia lyase* (*DcPAL*), *DcPR1*, *DcPR2*, *DcPR3*, *DcPR5*, as well as *DcAct* and *Dc18S*, were generated using RT-PCR on non-transformed carrot cDNA using specific primers (Wally et al. 2009b). Random primers were used to label the specific carrot RT-PCR products using [α-^{32}P] dCTP and Prime-A-gene kit (Promega) following the manufacturers protocols and used as radioactive DNA probes. Blots were washed several times for 20 min each; twice with 2× SSC at 60°C, twice with 1× SSC at 65°C and once with 0.25× SSC at 65°C, all containing 0.1% SDS. The blots were either exposed to X-ray film at −80°C for 3–7 days with an intensifying screen or for quantitative expression of carrot *PR* genes was assessed by exposing filters to phospho-storage screens (GE) and by subsequently scanning with the phospho-imaging system Si 445 (Molecular Dynamics, CA, USA). The signal was taken as the average of signal intensities normalized against both actin and 18S rRNA and standardized to the time zero points for the *35S::GUS* lines. For quantitative analysis, RNA extraction and blotting were repeated a minimum of three times.

Detection of hydrogen peroxide

Qualitative in vivo assessment of H_2O_2 production in carrot leaves was conducted by placing samples in 3′,3-diaminobenzidine (DAB) solution (1 mg ml^{-1}) overnight (Thordal-Christensen et al. 1997). The chlorophyll was cleared by boiling the leaves in solution of ethanol (95% v/v): glycerol:glacial acetic acid (3:1:1) for up to 45 min.

Elicitation for measurement of oxidative burst, 25 ml of 10-day-old carrot cell cultures were transferred to sterile 100 ml Erlenmeyer flask and agitated on a rotating shaker at 150 rpm. Xylenol orange was used to specifically monitor the production of H_2O_2 during the oxidative burst response in carrot cell suspension cultures. The hydroperoxides are reduced by ferrous ions in the acid solution to form a ferric–xylenol orange complex that was detected spectrophotometrically at 560 nm (Bindschedler et al. 2001; Gay et al. 1999). The H_2O_2 measurements were conducted at early time points up until 3 h after elicitation. Inhibitors of the oxidative burst including KCN (1 mM), NaN_3 (1 mM), L-cysteine (2.5 mM) (Sariri et al. 2006) and diphenylene iodonium (DPI 50 μM), were added 30 min prior to elicitation. Scavenging potential was measured by spiking the cultures with exogenous H_2O_2 up to 500 μM, and monitoring the rate of H_2O_2 reduction.

Quantification of lignin and soluble phenolics

Total lignin was extracted from carrot tissues from the alcohol-insoluble residue (AIR) and preferential solubilization through derivatization of lignin with a modified thiogylcolic acid method (Doster and Bostock 1988). Tap roots were examined for lignin content in the outer 2 mm peel comprised mainly of periderm and phloem tissues, using 0.5 g fresh weight of each tissue type. For suspension cultures, lignin was extracted from 50 mg (dw) of cells. All tissues were homogenized with a tissue polytron in absolute methanol; the cell material was pelleted and resuspended with five washes of methanol. The AIR was dried under vacuum overnight. Approximately 50 mg of AIR was incubated in 5 ml of 10% thiogylcolic acid in 2 N HCl at 95°C for 6 h. The soluble ligno-thioglycolic acid derivative (LTGA) product was precipitated by centrifugation for 10 min in a clinical centrifuge at 800*g*, and washed with water. The LTGA was suspended in 2 ml of 0.5 N NaOH through gentle shaking for 16 h at room temperature, the extract was cleared by centrifugation and the supernatant was acidified with 0.5 ml of concentrated HCl. The LTGA was precipitated following 4 h incubation at 4°C followed by 10 min centrifugation, and the pellet was washed twice with water before being resuspended in 0.5 ml of 0.5 N NaOH. The LTGA was quantified as A280 readings relative to the reading for the *35S::GUS* plants (Doster and Bostock 1988).The experiments were conducted three times with nine replicates for each of the treatments described.

Total soluble phenolics were measured from 100 μl of the methanol-soluble fraction. The extract was incubated with 1 ml of freshly prepared 2% (w/v) $NaCO_3$ for 5 min, to which 25 μl of undiluted Folin–Ciocalteu reagent was added (Vermerris and Nicholson 2006). The sample was mixed thoroughly and incubated for 30 min at 25°C. The absorbance at 750 nm was measured spectrophotometrically. Guaiacol was used to generate a standard curve.

Assessment of disease resistance

Greenhouse-grown carrot roots (20–24 weeks of age) were washed with water and placed in sealed plastic trays lined with moistened paper towels. Mycelial plugs of the fungal pathogen *Alternaria radicina* (provided by Dr. Barry M. Pryor, University of Arizona, Tucson, USA) from

2-week-old V8 agar cultures were placed evenly along the root (2–3 plugs per root). Lesion area was measured 10 days after inoculation (dai) as mm^2 and compared to *35S::GUS* lines. Three replicates consisting of four roots each were assayed. The experiment was conducted three times.

For assessment of powdery mildew resistance (*Erysiphe heraclei*), heavily infected leaves from non-transgenic plants were used as inoculum. Leaves from transgenic and non-transgenic *35S::GUS* control plants were harvested, washed in water, and cut into 3–4 cm segments and placed adaxial side up on water agar (6 g l^{-1}) containing benzimidazole (0.1 g l^{-1}) in 100 × 15 mm petri dishes, until 90% of the agar surface was covered. Spores were blown into a settling tower, allowed to settle over the tissues for 5 min, and the dishes were sealed and placed on the laboratory bench 22–25° C for 10 days. Newly formed sporulating colonies were counted per dish at 7 and 10 days, using a stereo dissecting microscope. The experiment was conducted three times, consisting of ten plates for each replicate.

Statistical analysis

Treatments were analyzed for significant differences using one-way ANOVA, followed by Tukey–Kramer HSD test and subsequently compared to the *35S::GUS* controls using Dunnett's control test using the JMP version 7 software (SAS institute 2008). LSD values at α <0.05 were used to determine significance.

Results

Effect of OsPrx114 on defense gene transcript levels

Constitutive transcript levels of genes *DcPR1*, *DcPR2, DcPR3* and *DcPR5* in line P23 were elevated compared to the *35S::GUS* control levels (Table 1). There was a marked increase in the transcript levels of *DcPR1, DcPR2, DcPR3* and *DcPR5* in cell cultures elicited with purified cell wall extracts of *S. sclerotiorum* (SS-walls), with no significant increase in *DcPAL* levels, after elicitation (Fig. 1); the maximum expression was observed 12–24 h after elicitation (Fig. 2). The *PR* genes were induced to a greater extent in the P23 cultures, with up to 30-fold increase in *DcPR1*(Fig. 2a), 45-fold increase in *DcPR2* (Fig. 2b), *DcPR3* (Fig. 2c) and a 20-fold increase with *DcPR5* (Fig. 2d) in comparison to 18-, 8-, 15- and 4.5-fold maximal induction for *DcPR1–DcPR5*, respectively in the *35S::GUS* controls (Fig. 2a–d). Application of the functional salicylic acid analog 2,6-dichloroisonicotinic acid (INA) (500 μM) or the phytohormone jasmonic acid (JA) (100 μM) enhanced defense gene expression in both P23 and *35S::GUS* lines, although the P23 line was more responsive, with more rapid expression of *DcPR1, DcPR2, DcPR3* and *DcPR5* compared to the *35S::GUS*. However, the degree of induction due to INA and JA was much lower than with the SS-walls treatment (Supplementary Figs. S1, S2).

Table 1 Fold transcript constitutive expression of *PR-1, PR-2, PR-3, PR-5* and *PAL* in *OsPrx114* line P23 relative to *35S::GUS* values normalized to both actin and 18 s rRNA

	PR-1	*PR-2*	*PR-3*	*PR-5*	*PAL*
P23	1.98*	2.25**	2.44	3.13*	1.23
Control	1.0	1.0	1.0	1.0	1.0
SE	0.26	0.23	0.75	0.59	0.18

Data presented is the mean of a minimum of 4 independent northern blots. Standard error of the mean is presented

* Significant LSD of <0.05; ** LSD of <0.01

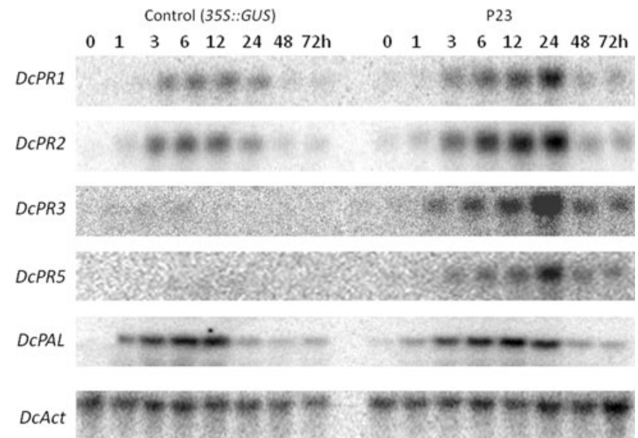

Fig. 1 Time-course induction of PR genes in transgenic carrot lines elicited by *S. sclerotiorum* cell wall fragments. Total RNA from transgenic *OsPrx114* expressing line P23 and control *35S::GUS* suspension cultures was extracted at 0, 1, 3, 6, 12, 24, 48 and 72 h after treatment with *S. sclerotiorum* cell walls (100 μg ml^{-1} dw). Northern blots were probed with the radio-labeled cDNA probes listed. Shown is a representative blot, from five separate replicates

Two non-specific peroxidase inhibitors, NaN_3 (1 mM) or KCN (1 mM), were added to the P23 cell cultures to determine if over-expression of peroxidase was responsible for the increase in defense gene transcript levels in transgenic tissues. Overall, defense gene transcript levels after addition of SS-walls were greatly reduced by adding either NaN_3 or KCN (Fig. 3).

To determine if changes in gene transcript levels were due to increased levels of extracellular H_2O_2, we investigated the effect of adding exogenous H_2O_2. *35S::GUS* control lines responded weakly to 250 μM, with a slight increase in *DcPR1*, *DcPR2* and *DcPR5* expression. Much larger increases in transcript levels of these genes were observed after H_2O_2 addition in line P23 (Table 2). This increase, however, was less than that observed following SS-walls elicitation (Table 2).

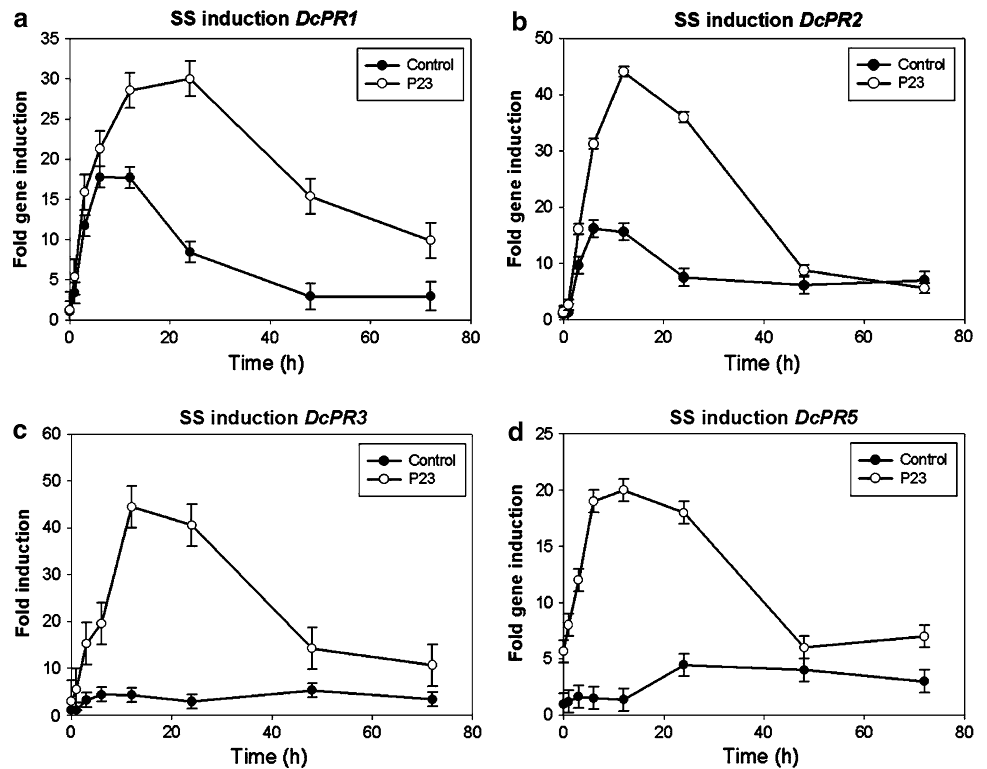

Fig. 2 Induction of various carrot pathogenesis-related (*DcPR*) genes following induction of suspension cultures using purified *S. sclerotiorum* cell wall fragments (SS-walls) at various time points, comparing *OsPrx114* over-expressing P23 line against *35S::GUS* control line. Induction was quantified from Northern blots relative to 0 h transcript level for the control lines for **a** *DcPR1*, **b** *DcPR2*, **c** *DcPR3* and **d** *DcPR5*. Transcripts were standardized to both 18S and Actin levels. The experiment was repeated 5 times, and the vertical *error bars* represent the standard error of the mean

Detection of H_2O_2

Extracellular peroxidases can generate H_2O_2 through the hydroxylic cycle, which is an alternative to the normal peroxidative cycle (Passardi et al. 2005). Previous over-expression of a sweet potato peroxidase (*IbPrx04*) in transgenic tobacco showed high levels of constitutive H_2O_2 accumulation in leaves following staining with 3′,3-diaminobenzidine (DAB) (Kim et al. 2008). We therefore attempted to determine if H_2O_2 was enhanced in the P23 transgenic carrot leaves. There was no noticeable difference in the accumulation of H_2O_2 in leaves (Supplementary Fig. S3) or roots (not shown) of line P23 compared to the *35S::GUS* plants. A similar low staining of polymerized DAB was observed near the wound sites in both *35S::GUS* and P23 lines.

Control *35S::GUS* cells treated with SS-walls at a concentration of 100 mg l^{-1} exhibited a rapid and reproducible oxidative burst response when assayed with xylenol orange (Fig. 4a). In contrast, when the P23 cells were treated with SS-walls, the oxidative burst response was negligible and there was no apparent increase in H_2O_2 levels (Fig. 4a).

To determine if the H_2O_2 generated through the oxidative burst response was due to activity of peroxidases or NADH-oxidases, inhibitors were applied to the cell cultures. NAD(P)H oxidases are highly sensitive to inhibition by low concentrations of DPI, whereas DPI concentrations greater than 100 μM are required for peroxidase inhibition (Davies et al. 2006). Peroxidase activity was inhibited in P23 by addition of NaN_3 and KCN, which do not affect NADH-oxidases. The oxidative burst response was lowered slightly following DPI treatment, with the *35S::GUS* cells showing slightly lower extracellular H_2O_2 across all time points (Fig. 4b). The addition of either NaN_3 or KCN resulted in a near

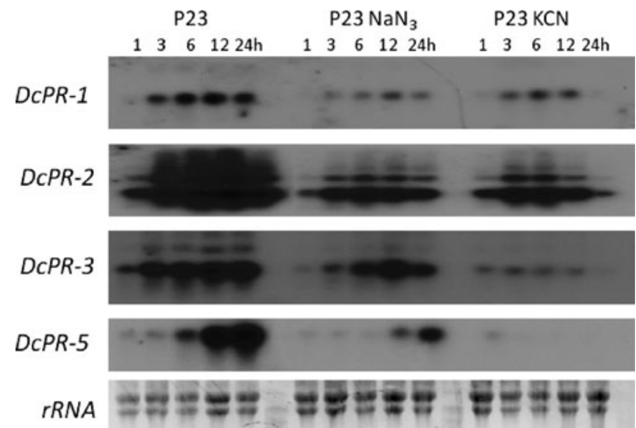

Fig. 3 Time-course induction of PR genes in transgenic P23 carrot lines elicited by *S. sclerotiorum* cell wall fragments, comparing effect of inhibitors. Total RNA from suspension cultures of transgenic *OsPrx114* expressing line P23, with or without 1 mM NaN_3 or 1 mM KCN was extracted at 0, 1, 3, 6, 12 and 24 h after treatment with *S. sclerotiorum* cell walls (100 μg ml^{-1} dw). Northern blots were probed with the radio-labeled cDNA probes listed

Table 2 Fold transcript expression of *DcPR1*, *DcPR3* and *DcPR5* in *OsPrx114* expressing line P23 relative to *35S::GUS* values following induction with SS-walls or H_2O_2 (500 μM), normalized to both actin and 18 s rRNA

Time (h)	Treatment											
	SS-walls						H_2O_2					
	35S::GUS			P23			Control			P23		
	PR1	*PR3*	*PR5*	*PR1*	*PR3*	*PR5*	*PR1*	*PR3*	*PR5*	*PR1*	*PR3*	*PR5*
6	17.8	6.4	1.6	21.3	19.1	12.1	1.2	1	1.0	3.3	1.8	4.8
12	17.9	5.2	1.5	28.2	44.8	19.0	1.9	1.4	1.11	3.9	3.0	3.9
24	8.8	7.67	4.5	30.1	40.2	20.3	2.9	1.3	1.10	13.7	15.0	5.99
48	2.8	6.71	4.4	15.4	14.6	6.2	1.7	1.5	1.2	7.9	9.2	4.06
SE	1.32	1.51	1.02	2.25	4.45	1.7	0.67	0.42	0.39	1.4	1.52	1.1

Data represented is the mean of a minimum of 6 independent northern blots. Standard error of the mean for the experiment is presented

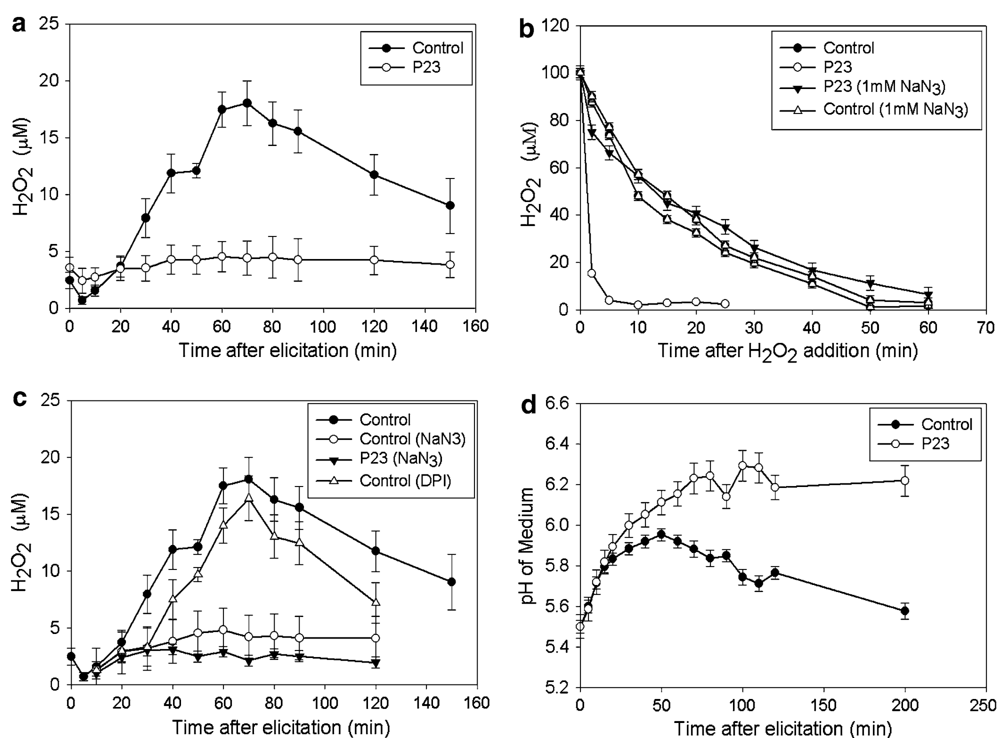

Fig. 4 Biochemical responses in suspension cultures of *35S::GUS* controls and transgenic P23 carrot lines. **a** Treatment with 100 μg ml^{-1} glucose equivalents of *S. sclerotiorum* cell wall elicitor. Production of H_2O_2 was measured using a xylenol orange assay. **b** Rate of H_2O_2 consumption following addition of 100 μM H_2O_2, and 1 mM NaN_3 pre-applied to line P23. **c** Inhibitor treatment with 1 mM NaN_3 and diphenylene iodonium (DPI) 50 μM. **d** Changes in pH of culture media following elicitation with *S. sclerotiorum* cell wall fragments (100 μg ml^{-1}). *Vertical error bars* represent the standard error of the mean from 5 replicates from a minimum of independent 4 experiments

abolition of the oxidative burst response, with the H_2O_2 levels in both *35S::GUS* controls and P23 lines detected at basal levels (Fig. 4b). Since both KCN and NaN_3 are highly oxidized and non-specific inhibitors of peroxidase, we also examined L-cysteine as an alternative inhibitor (Sariri et al. 2006). L-Cysteine (2.5 mM) reduced the oxidative burst by nearly 80% in the *35S::GUS* lines and did not alter the levels found in line P23 (not shown). These findings indicate that the extracellular oxidative burst response in carrot cells was due mainly to peroxidase activity and not NADH-oxidases.

H_2O_2 scavenging potential

When suspension cultures were initially spiked with 25 μM H_2O_2, levels of H_2O_2 were undetectable after 30 s in the P23 line, indicating extremely rapid H_2O_2 consumption (data not shown). Additional H_2O_2 experiments were

conducted with higher concentrations of H_2O_2. When 100 μM of H_2O_2 was applied to the P23 line, the levels were reduced by 80% within 2 min and were at basal levels after 5 min (Fig. 4c). In contrast, the *35S::GUS* controls exhibited a more gradual reduction in H_2O_2, with an 80% reduction after 40 min and a return to basal levels after 50 min. NaN_3 was applied to the cells prior to H_2O_2 application to suppress peroxidase activity and to determine if heightened peroxidase levels were responsible for the increased scavenging. This resulted in the P23 lines removing H_2O_2 at levels comparable to the *35S::GUS* (Fig. 4c), and similar results were obtained using KCN (not shown). The ability of *35S::GUS* lines to consume H_2O_2 was largely unaffected by the presence of either NaN_3 or KCN.

To determine if the oxidative burst response was reduced in the P23 cells and was not a consequence of the heightened scavenging ability, we examined the effect on extracellular akalinization in suspension cultures when elicited with SS-walls. The response of line P23 was identical to the *35S::GUS* line during the initial 30 min of the oxidative burst response, with a rapid increase in the pH of the growth medium from 5.3 to over 5.8 (Fig. 4d). After the initial phase, the rate of akalinization slowed in the *35S::GUS*, peaking at 60 min before gradually decreasing. In contrast, the pH continued to increase in the P23 suspension cultures, peaking at a pH of 6.3 at 120 min and maintaining a pH higher than 6.0 past 200 min (Fig. 4d).

Assessment of disease resistance

The *OsPrx114* over-expressing line P23 was previously found to be highly resistant to the foliar necrotrophic pathogens *B. cinerea* and *S. sclerotiorum* (Wally et al. 2009a). Resistance to the root pathogen *A. radicina* and the biotrophic foliar pathogen *E. heraclei* was further investigated in this study. The tap roots of line P23 were highly resistant to infection by *A. radicina* (Fig. 5a), and the total lesion area was reduced by up to 80% compared to the *35S::GUS* roots (Fig. 6a). Furthermore, the lesions were largely superficial on the P23 roots, with *A. radicina* producing mycelia only on the surface of the root (Fig. 5a, b). The degree of fungal penetration was also greatly reduced in the P23 line (Fig. 5b). These differences were very apparent when examined microscopically in roots stained for the presence of lignin (Fig. 5c, d). The *35S::GUS* roots had very low levels of lignin (detected with phloroglucinol) and tissue degradation and infection proceeded beyond the periderm (Fig. 5c). The P23 tap roots produced a thick lignin layer directly below the infected area, preventing further penetration and colonization of the tissues (Fig. 5d). There was no fungal mycelium seen below the lignin layer in P23, while a high degree of tissue necrosis with visible mycelia was present beneath the thin lignin layer in the *35S::GUS* roots. Interestingly, the peridermal zone of high lignin did not appear to be constitutively formed, since areas away from the lesion did not have detectable lignin using phloroglucinol (not shown). The P23 leaves and petioles exhibited comparable susceptibility towards the biotrophic pathogen *E. heraclei* as the *35S::GUS* tissues (Fig. 5b).

Determination of lignin levels

Lignin levels were measured in the tap roots in the outermost (∼2 mm) peel, consisting of periderm and inner layer of vascular tissue comprised mainly of the phloem

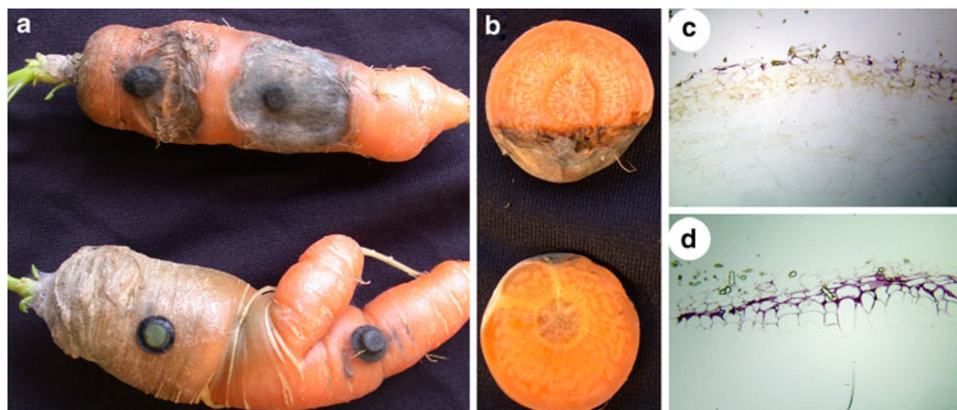

Fig. 5 Resistance of transgenic P23 carrot roots to *A. radicina* 10 days after inoculation, compared to *35S::GUS* roots. **a** Typical large developing lesions in *35S::GUS* (*upper root*), compared to relatively lesion free P23 root (*bottom root*). **b** Cross-section through the lesion area. The deep necrotic lesion is apparent in the *35S::GUS* (*top*), while the lesion is fairly superficial on P23 root (*bottom*). Low magnification micrograph of the *35S::GUS* (**c**) and P23 root (**d**), taken from the margin of the infected area and stained with phloroglucinol to detect lignin (*violet red zone of cells*)

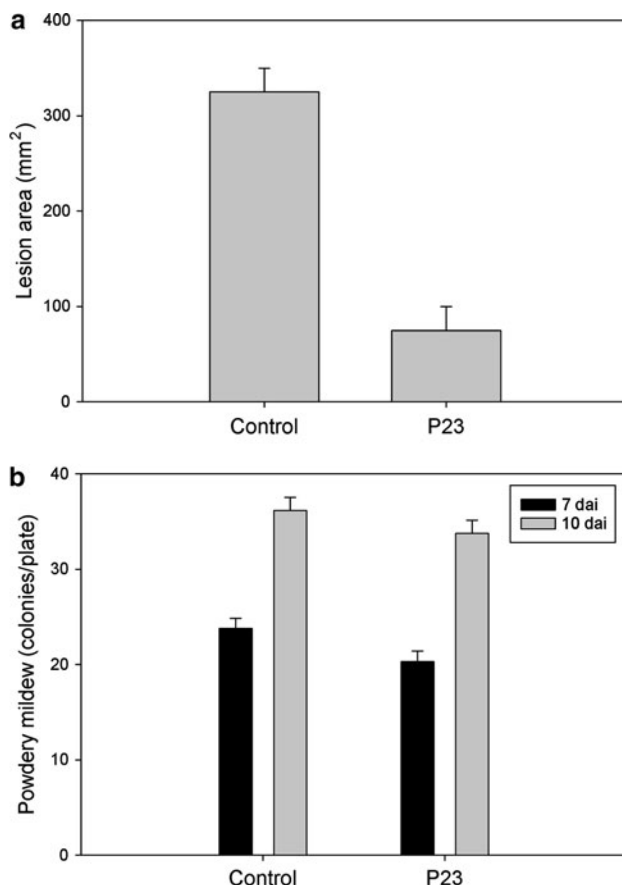

Fig. 6 Response of transgenic *OsPrx114* expressing carrots line P23 to black rot and powdery mildew infection. **a** Resistance of harvested tap roots to black rot (*A. radicina*) 10 dai measured as the average total area of each lesion 10 days after inoculation. **b** Response of the P23 line compared to *35S::GUS* on excised leaflets inoculated with *E. heraclei* spores on water agar plates, based on the number of newly formed sporulating colonies at 7 and 10 days after inoculation. Three replicates of 10 plates were counted for each line. *Error bars* represent standard error of the means, LSD <0.05

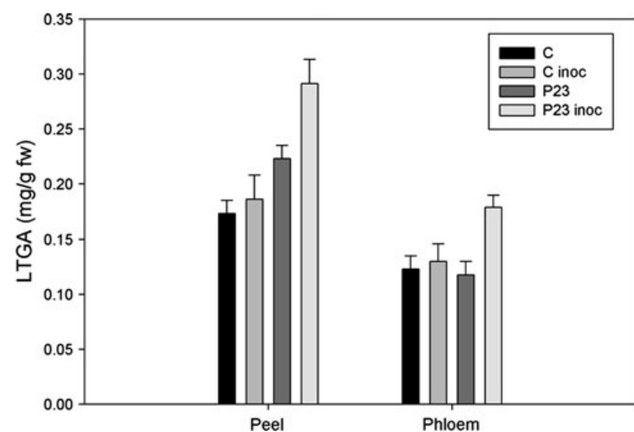

Fig. 7 Total derivatized lignin-thioglycolic acid complex content in roots of *35S::GUS* control and peroxidase over-expressing carrot line P23. Samples were taken from the outer 2 mm peel or from the secondary phloem in uninoculated and inoculated roots 3 days after inoculation with *A. radicina*. *Vertical error bars* indicate standard error of the mean from 9 replicate samples for each treatment, LSD <0.05

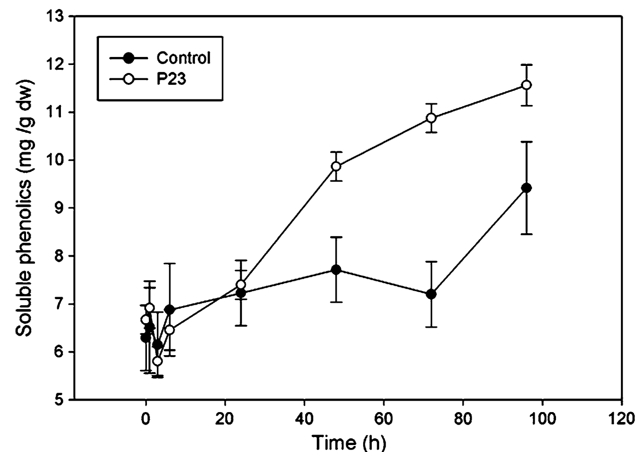

Fig. 8 Soluble phenolic compounds extracted from suspension cultures of *35S::GUS* or P23 peroxidase expressing lines following inoculation with *S. sclerotiorum* cell wall fragments. *Error bars* indicate the standard error of the mean for each time point

parenchyma. Additionally, lignin levels were measured in suspension cultures under non-induced and SS-walls treated conditions. The P23 line had 20% greater total lignin levels in the peel under non-induced conditions than the *35S::GUS* control line, with no increase in lignin in the phloem tissues (Fig. 7). Lignin levels were increased further in the P23 roots when challenged with *A. radicina*, in both the peel (by 30%) and phloem tissues (by 50%) (Fig. 7). The *35S::GUS* roots had no significant increase in lignin due to infection (Fig. 7). Suspension cells of line P23 had lignin levels similar to that of *35S::GUS* cells, both when non-induced or treated with SS-walls after 5 days (data not shown).

Since the level of lignification was enhanced in the presence of a pathogen, we also examined the level of soluble phenolic compounds to determine if these were increased or if the levels were limited to lignin deposition during infection. Total soluble phenolics were measured in both *35S::GUS* and P23 taproots and leaf tissue, in the absence and presence of the pathogen *S. sclerotiorum*. There was a slight increase in the phenolic levels during infection; however, the variation was very high and no significant differences were detected between the *35S::GUS* and P23 lines (data not shown). Using the suspension culture cells, with defense response elicited by SS-walls, there was also no increase in the phenolic levels in P23 cells compared to *35S::GUS* during the first 24 h. However, there was a significant increase in the production of phenolics after 48 h in line P23, which continued to increase to 96 h (Fig. 8).

Discussion

Peroxidases have been reported to have important roles in tolerance to abiotic and biotic stresses, as well in various aspects of plant growth and development, including generating and utilization of H_2O_2 (Passardi et al. 2005; Johrde and Schweizer 2008; Cosio and Dunand 2009). H_2O_2 is involved in multiple ways in plant defense responses to pathogens. At low concentrations, H_2O_2 can function as a signaling molecule, involved in gene regulation and activation of secondary defense pathways (Bolwell 1999; Neill et al. 2002a, b). At higher concentrations, H_2O_2 has been associated with cell wall modification, the hypersensitive response, or even directly inhibiting pathogens (Lamb and Dixon 1997). However, the role of H_2O_2 in pathogen defense is complex, since there is evidence to suggest that H_2O_2 accumulation can be beneficial for colonization of plant tissue by necrotrophic pathogens (Govrin and Levine 2002), while being suppressive to biotrophic pathogens. In contrast, the local production of H_2O_2 has been associated with enhanced resistance towards the necrotrophic fungus *B. cinerea* in tomato plants (Asselbergh et al. 2007). Tobacco plants over-expressing a sweet potato peroxidase *IbPrx04* had up to fivefold the basal H_2O_2 levels in leaves (Kim et al. 2008). Similarly, over-expression of a sweet pepper peroxidase *CaPrx02* in Arabidopsis was associated with increased H_2O_2 levels (Choi et al. 2007). In contrast, *OsPrx114* over-expressing carrot organs had no detectable increase in H_2O_2 (Supplemental Fig S3). Apoplastic oxidative burst response is typically controlled either through cell wall bound peroxidases as in French bean (Bindschedler et al. 2001) and Arabidopsis (Bindschedler et al. 2006; Davies et al. 2006) or through the function of NADH-oxidases as in rose cells (Bolwell et al. 1998). The oxidative burst response in carrot tissues elicited by SS-walls appears to be controlled mainly by cell wall bound peroxidases rather than NADH-oxidases, since the burst was effectively eliminated following addition of NaN_3, KCN or L-cysteine, and only marginally reduced by adding DPI (Fig. 4). Interestingly, the P23 line had no detectable accumulation of H_2O_2 in culture media. Extracellular alkalization is a key component of the oxidative burst, independent of H_2O_2 levels (Bolwell et al. 1998). In tissue-cultured cells of line P23, alkalinization of the medium continued at a longer and slightly stronger rate than in the *35S::GUS* line, indicating the oxidative burst response was not inhibited rather there was no accumulation of H_2O_2 (Fig. 4d). Additionally, cells of P23 were able to rapidly remove high levels of exogenous H_2O_2, and this ability was effectively eliminated by adding a peroxidase inhibitor (Fig. 4c). These findings may indicate that *OsPrx114* over-expression in the P23 line caused a rapid utilization or scavenging of H_2O_2 generated during the oxidative burst response. OsPrx114 appears to function by rapidly removing the available H_2O_2 in the apoplastic space through oxidation of available phenolic metabolites rather than as a H_2O_2 generating peroxidase, operating in a similar fashion to the LePrx06 peroxidase from tomato (Coego et al. 2005).

The signal transduction pathway downstream of H_2O_2 may be modulated directly or indirectly through the activity of peroxidases, potentially through interactions with extracellular signaling recognition via protein kinases (Lamb and Dixon 1997). Enhanced constitutive expression of many defense-related genes was observed in transgenic tobacco over-expressing a peroxidase gene (Kim et al. 2008). The level of expression was reduced significantly by application of either NaN_3 or KCN, which was attributed to a reduction in the overall level of H_2O_2 in the tobacco cells (Kim et al. 2008). There was a slight constitutive enhancement of defense gene expression in P23 carrot cells, but the level of expression was dramatically increased when the cells were elicited with SS-walls, indicating that these tissues were primed for a more rapid and intense response to pathogens (Table 1). While the addition of NaN_3 or KCN lowered the enhanced induction of PR gene transcripts, the transcript levels were still significantly higher than the induced *35S::GUS* control levels (Fig. 3). The application of exogenous phytohormones INA, JA (Supplemental figures S1, S2) or H_2O_2 (Table 2) resulted in marginal increases in transcripts in both *35S::GUS* and P23 lines; however, the extent of transcript induction was a fraction of that observed with the SS-walls. The use of complex oligosaccharides found in fungal cell walls has been shown to induce multi-factorial signaling events in plants, generally resulting in much stronger levels of gene induction than a single hormone (Leitner et al. 2008; Jayaraj et al. 2009) and ultimately providing an induced resistance response.

Lignin is a strong structural polymer which is very difficult for pathogens to penetrate or degrade (Quiroga et al. 2000). OsPrx114 has been identified as a putative lignin-forming peroxidase (Hilaire et al. 2001). Previously, we found that *OsPrx114* expressing carrot petioles accumulated 40% more lignin than *35S::GUS* controls without pathogen challenge, which further increased to 70% in the presence of *S. sclerotiorum* (Wally et al. 2009a). Since excess lignin can potentially reduce the palatability of root tissue, the lignin levels of mature tap roots in line P23 were measured and compared to *35S::GUS* roots. Lignin levels in the peel of the transgenic root (consisting mainly of periderm) were enhanced similar to that observed in the petioles, both constitutively and following pathogen challenge. However, there was no constitutive increase in the lignin levels in the phloem tissues, with increases only

observed during pathogen challenge. Since the peel is generally removed before carrot consumption, the increased lignin in the peel or petioles should not be overly detrimental to the palatability (Fig. 7). There was also no increase in the lignin levels in P23 suspension cultures constitutively or induced with SS-walls, possibly indicating a lack of lignin precursors, which limits the lignin levels in fleshy tissues. Increases in soluble phenolic compounds have been associated with peroxidase over-expression in tobacco (Lagrimini 1991; Kim et al. 2008); however, the mechanism of this induction is unknown. When quantified, the *OsPrx114* over-expressing carrot line did not exhibit elevated phenolic content in roots, leaves or suspension cultures. There was a gradual increase in the total phenolic content in P23 suspension cultures after elicitation with SS-walls after 24 h and continuing past 96 h compared to *35S::GUS* (Fig. 8). This increase in phenolic levels suggests that peroxidase over-expression may allow for more rapid responses of the phenylpropanoid pathway, rather than constitutive induction.

The taproots of P23 carrots had greatly enhanced resistance to the necrotrophic pathogen *A. radicina*, showing up to 80% reduction in lesion area (Figs. 5, 6). This level of resistance is high, comparable to foliar resistance to *S. sclerotiorum* or *B. cinerea* (Wally et al. 2009a). The susceptibility of line P23 to powdery mildew differs from what was reported in wheat (Altpeter et al. 2005; Johrde and Schweizer 2008), barley (Johrde and Schweizer 2008) and tobacco (Kim et al. 2008), where over-expression of a peroxidase gene led to enhanced resistance to powdery or downy mildews. The P23 line did not show a constitutive increase in H_2O_2 nor the high levels of PR gene expression reported in transgenic tobacco (Kim et al. 2008). The level of peroxidase expression was also greater in barley and tobacco, in excess of 40-fold (Johrde and Schweizer 2008; Kim et al. 2008) compared to carrot, which may have led to reduced mildew infection, as reported in wheat (Altpeter et al. 2005).

In summary, we have shown that line P23 expressing *OsPrx114* had reduced accumulation of H_2O_2 when undergoing an oxidative burst response. In addition, heightened induced expression of defense-related gene transcripts and taproot lignin levels were observed. These findings suggest that constitutive *OsPrx114* expression is involved in multiple signaling and defense responses in carrot tissues.

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