Planta (2008) 227:867–881
DOI 10.1007/s00425-007-0663-3

ORIGINAL ARTICLE

Overexpression of sweetpotato *swpa4* peroxidase results in increased hydrogen peroxide production and enhances stress tolerance in tobacco

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Received: 15 July 2007 / Accepted: 29 October 2007 / Published online: 16 November 2007

Abstract Plant peroxidases (POD) reduce hydrogen peroxide (H_2O_2) in the presence of an electron donor. Extracellular POD can also induce H_2O_2 production and may perform a significant function in responses to environmental stresses via the regulation of H_2O_2 in plants. We previously described the isolation of 10 POD cDNA clones from cell cultures of sweetpotato (*Ipomoea batatas*). Among them, the expression of the *swpa4* gene was profoundly induced by a variety of abiotic stresses and pathogenic infections (Park et al. in Mol Gen Genome 269:542–552 2003; Jang et al. in Plant Physiol Biochem 42:451–455 2004). In the present study, transgenic tobacco (*Nicotiana tabacum*) plants overexpressing the *swpa4* gene under the control of the CaMV 35S promoter were generated in order to assess the function of *swpa4 in planta*. The transgenic plants exhibited an approximately 50-fold higher POD specific activity than was observed in control plants. Both transient expression analysis with the swpa4-GFP fusion protein and POD activity assays in the apoplastic washing fluid revealed that the swpa4 protein is secreted into the apoplastic space. In addition, a significantly enhanced tolerance to a variety of abiotic and biotic stresses occurred in the transgenic plants. These plants harbored increased lignin and phenolic content, and H_2O_2 was also generated under normal conditions. Furthermore, they showed an increased expression level of a variety of apoplastic acidic pathogenesis-related (PR) genes following enhanced H_2O_2 production. These results suggest that the expression of *swpa4* in the apoplastic space may function as a positive defense signal in the H_2O_2-regulated stress response signaling pathway.

Keywords Acidic PR genes · Apoplastic space · Environmental stresses · Hydrogen peroxide · *Ipomoea* · *Nicotiana* · Peroxidase

Abbreviations

DAB	3′,3-Diamonobenzidine
GFP	Green fluorescent protein
MV	Methyl viologen
PR	Pathogenesis related
POD	Peroxidase

Introduction

The secretory class III peroxidases (POD, EC 1.11.1.7) catalyze the reduction of H_2O_2 via the transport of electrons to various donor molecules, including phenolic compounds, lignin precursors, and auxin (Hiraga et al. 2001; Passardi et al. 2005). They have been implicated in a broad range of physiological processes, including lignification, suberization, auxin metabolism, the cross-linking of cell wall proteins, defense against pathogenic attack, and a variety of

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abiotic stress tolerances (Hiraga et al. 2001; Passardi et al. 2005). Recent findings have indicated that extracellular POD may also generate H_2O_2 as a consequence of its reactions (Bolwell et al. 2002; Mika et al. 2004). It has been suggested that H_2O_2 production may play important roles in response to a variety of abiotic and biotic stresses in plants (Kawano 2003; Mika et al. 2004). Plant POD has been detected in a large family of isoenzymes in a variety of higher plants (Duroux and Welinder 2003). However, the inherent complexity of the physiological processes in which POD isoenzymes are involved makes understanding the specific function of each of these enzymes rather difficult. One way to ascertain physiological functions is to generate transgenic plants with enhanced or suppressed expression of a specific POD gene.

In our previous studies, we established an efficient POD production system from suspension cultures of sweetpotato (*Ipomoea batatas*; Kim et al. 1994; Kwak et al. 1995). Ten POD cDNA clones were isolated from sweetpotato cell cultures, and their expression profiles were determined as the initial step toward understanding the physiological functions of each POD gene in response to a variety of environmental stresses (Huh et al. 1997; Kim et al. 1999, 2000; Park et al. 2003; Jang et al. 2004). In the ten POD cDNAs, the transcript levels of *swpa4* were shown to have been markedly increased under a variety of stress conditions, including methyl viologen (MV), H_2O_2, salt, chilling, wounding, and pathogenic bacteria (Park et al. 2003; Jang et al. 2004).

In this study, we demonstrate that transgenic tobacco plants overexpressing *swpa4* showed increased H_2O_2 production followed by the up-regulation of multiple apoplastic acidic PR genes. The *swpa4* transgenic plants manifested significantly enhanced tolerance to a variety of abiotic and biotic stresses in the H_2O_2-regulated stress response signaling pathway.

Materials and methods

Constructs and plant transformation

In order to overexpress the *swpa4* gene in tobacco plants, we constructed a plasmid for the sense expression of the *swpa4* gene by cloning it into the *Bam*HI site of pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA, USA). The coding region of the *swpa4* gene was introduced behind the CaMV 35S promoter in the pCAMBIA2300 plant expression vector. The constructs were verified via sequencing. The CaMV35S::*swpa4* and control (pCAMBIA2300 vector only) plasmids were introduced into *Agrobacterium tumefaciens* EHA 105, which was transformed into tobacco (*Nicotiana tabacum* cv. Xanthi) via an *Agrobacterium*-mediated transformation method (Kim et al. 2003). The kanamycin-resistant plants were selected and permitted to self-fertilize. T_1 seeds were germinated in a basic MS (Murashige and Skoog 1962) medium containing 150 mg l^{-1} kanamycin, and the selected seedlings were cultivated in a greenhouse.

Northern-blot analysis

For Northern-blot analysis, the total RNA was isolated from tobacco plants using TRizol reagent (Invitrogen). Total RNA, 30 μg, was denatured at 70°C and separated on 1.2% agarose-formaldehyde gel, then transferred onto a Zeta-probe GT membrane (Bio-Rad, Hercules, CA, USA). The blot was hybridized to a ^{32}P-labeled full-length *swpa4* cDNA probe.

Subcellular localization of *swpa4*

The coding region of *swpa4* was PCR amplified using the following primers with a *Bam*HI restriction site: for *swpa4*, 5′-CGGATCCATGGCTTCCTTTGTCACTCG-3′ and 5′-GGGATCCACATGGATGCAACTGTG-3′. The amplified DNA was initially cloned into the pCR2.1-TOPO vector (Invitrogen), after which the *Bam*HI fragment was ligated into the 35S::*GFP* vector, in which the swpa4 was in-frame fused with GFP at the C-terminus, thereby yielding swpa4::GFP. The resultant construct was then transiently introduced into onion epidermal cells with a biolistic particle delivery system (Bio-Rad), as was previously described by Shieh et al. (1993). After 12–48 h of incubation at 25°C, the subcellular distribution of the swpa4::GFP fusion proteins was evaluated via fluorescence microscopy (Axioskop, Zeiss, Jena, Germany).

Sample preparation and determination of POD activity

The frozen leaves of transgenic plants were ground in liquid nitrogen and homogenized in 0.1 M potassium phosphate buffer, at a pH of 6.0. After 15 min of centrifugation at 12,000*g* at 4°C, the supernatant was utilized to determine the soluble POD activity. The sample for the cell wall-bound POD activity assay was extracted as described by Mitsuya et al. (2006) with slight modifications. The washed pellet was then shaken with 3 volumes of 1 M NaCl in an extraction buffer for 2 h at room temperature and centrifuged for 10 min at 12,000*g* at 4°C. The supernatant was then employed for the cell wall-bound POD activity assay. Apoplastic washing fluid was extracted via a slight modification of the vacuum infiltration/centrifugation technique (Fecht-Christoffers et al. 2003). The leaves were cut from the tobacco plant and infiltrated with deionized water. The pressure was reduced to 70 kPa (5 min) using a water jet

pump, followed by 5 min of slow relaxation. The apoplastic washing fluid was recovered via 5 min of centrifugation at 1,000*g* at room temperature. Protein concentrations were determined with Bradford (1976) protein assay reagent (Bio-Rad). POD activity was assayed in accordance with the method developed by Kwak et al. (1995) using pyrogallol and H_2O_2 as substrates.

Native polyacrylamide gel electrophoresis

Native polyacrylamide gel electrophoresis (PAGE) of POD was performed on a 7.5% gel at 120 V at 4°C for 4 h (Beauchamp and Fridovich 1971). Isoelectric focusing (IEF) was conducted in 6.7% polyacrylamide gel with 4% ampholyte in a pH gradient from 3 to 10. The POD gel assays were conducted with the soluble leaf extracts, in accordance with the protocols established by Kim et al. (1994). After electrophoresis, the POD gel was stained with 1% benzidine and 1.5% H_2O_2.

Abiotic stress treatment and tolerance assays

MV, H_2O_2, mannitol, and NaCl damage were analyzed using the leaf discs from tobacco plants. Seven leaf discs (16 mm diameter) collected from the third leaves of T_3 plants were transferred to 5.0 cm Petri-dishes containing 10 ml of each solution at various concentrations (MV: 0, 2, 5 μM; H_2O_2: 0, 200, 400 mM; mannitol: 0, 600, 800 mM; NaCl: 0, 200, 400 mM). For dehydration treatment of the whole plants, 2-month-old transgenic plants were not watered for 8 days and then watered for 8 days. For salt treatment, the transgenic plants were irrigated with 300 mM NaCl solution for 10 days and then with water for 6 days. The conductivity of the decanted MV and H_2O_2 solutions was determined using an ion conductivity meter (Kwon et al. 2003). The chlorophyll (Chl) contents were analyzed with a UV spectrophotometer (Porra et al. 1989). The recovery of photosynthetic activity from the dehydration treatments was estimated by a Chl fluorescence determination of photochemical yield (*Fv*/*Fm*). The *Fv*/*Fm* content represents the maximal yield of the photochemical reaction on PSII, and was determined with a portable Chl fluorescence meter (PAM 2000). Lipid peroxidation levels in mannitol-treated leaf discs were assessed using the thiobarbituric acid test for malonaldehyde (Peever and Higgins 1989).

Biotic stress treatment and resistance assays

The fungal pathogen that causes tobacco black shank disease (*Phytophthora parasitica* var *nicotianae*, KACC 40164) was grown on V-8 juice agar plates. After the fungal mycelia had spread throughout the plate, a plug of medium containing the fungal mycelia was excised with a 3 mm cork borer, as described by Guo et al. (2004). Four leaves were utilized for each transgenic line, and the challenged leaves were maintained in a plastic dish with water-soaked filter paper at 25°C under a 16 h light/8 h dark photocycle. Cell death was visualized in the fungus-inoculated leaves via lactophenol-trypan blue staining, as described by Koch and Slusarenko (1990). Insecticidal activities were evaluated with 2-month-old plants against tobacco cutworm (*Spodoptera litura*) larvae at different developmental stages. For the first instar larvae, the insect eggs were placed on the tobacco leaves, allowing the hatched larvae to feed on the plants. In order to test the second to fourth instar larvae, 50 larvae were placed on the plants and the larval growth and survival rates were monitored for 4 days.

Histochemical staining analysis

For the localization of POD activity in the tobacco tissues, hand-cut transverse sections from tobacco stems were analyzed. POD activity staining was conducted as described by Lavid et al. (2001). The cut tobacco stems were incubated in 50 mM sodium phosphate at neutral pH, containing 5.3 mM guaiacol and 30 mM H_2O_2. For phenolics staining, after stem cutting, ferric chloride (50 mM in 0.1 M HCl) and 8 mM $K_3Fe(CN)_6$ were mixed in equal proportions prior to dipping (Lavid et al. 2001). The phenols produced a blue color at room temperature. For lignin staining, the stems were incubated overnight in 70% ethanol containing 1% phloroglucinol (Mlickova et al. 2004). After rinsing them in distilled water, the stems were bathed in 50% HCl.

Determination of the lignin and phenolic contents

The soluble phenolics were extracted and determined in accordance with the methods established by Stadnik and Buchenauer (2000). The methanol extracts were centrifuged for 10 min at 12,000*g* and the supernatants were utilized for the Folin-Ciocalteau assay and HPLC analysis. The remaining leaf tissue was dried for 48 h at 60°C, powdered and utilized for the quantification of lignin content. The contents of the soluble phenolics were determined using *p*-coumaric acid as a standard at A_{725}. Lignin contents were determined via thioglycolic acid assays (Stadnik and Buchenauer 2000; Hatfield and Fukushima 2005). For 4 h at 100°C, 50 mg of dry powdered samples were treated with 0.5 ml of a 1:10 mixture of thioglycolic acid and 2 N HCl. After two washings with water, the lignothioglycolic acid was extracted from the pellet with 1 ml of 0.5 N NaOH for 18 h. The lignin contents were determined at A_{280} using a lignin standard (alkali, 2-hydroxypropyl ether, Aldrich). Qualitative and quantitative analyses of the phen-

olics were conducted via reverse-phase HPLC (Talcott and Howard 1999). A volume of 20 µl of the sample was injected into a Shimadzu (Tokyo, Japan) HPLC system fitted with a 3.9 × 280 mm XTerra C_{18} column (Waters, Milford, MA, USA). The phenolic acids were detected at A_{280} using a Shimatsu SDP-10A UV-visible detector. Mobile phase A contained 98% water and 2% acetic acid, and mobile phase B contained 68% water, 30% acetonitrile, and 2% acetic acid. A linear gradient from 0 to 30% of mobile phase B for 30 min at 1 ml min^{-1} was used. The system column was washed with 100% mobile phase B and equilibrated with 100% mobile phase A, prior to the next sample injection. The identity of the phenolic acids was verified via co-chromatography on HPLC with authentic standards (Sigma, St Louis, MO, USA), and quantification was conducted using a standard curve in the 0–2 µg range of a standard mixture, as described by Sgherri et al. (2004). Chromatogram analysis was conducted with SCL-10 AVP software (Shimadzu).

H_2O_2 assay and POD inhibitor treatment

In order to visualize the H_2O_2, tobacco leaves were placed in 1 mg ml^{-1} of 3′,3-diaminobenzidine (DAB) solution. The samples were then incubated for 8 h in a growth chamber, and the Chl was cleared at 80°C for 2 h in 80% ethanol (Thordal-Christensen et al. 1997). The H_2O_2 contents were assessed with xylenol orange, in which H_2O_2 is reduced by ferrous ions in an acidic solution that forms a ferric product–xylenol orange complex, which was detected at A_{560} (Bindschedler et al. 2001). H_2O_2 measurements were expressed as relative values. For the POD inhibitor treatment, the leaves were detached from the tobacco plants and infiltrated for 12 h with 100 mM KCN or 200 mM NaN_3.

RT-PCR Analysis

Total RNA was isolated from tobacco leaves with TRIzol reagent (Invitrogen). RT-PCR amplification was conducted using an RT-PCR kit (Promega, Madison, WI, USA) in accordance with the manufacturer's instructions. Total RNA, 1 µg, was utilized for the generation of first-strand cDNA using MMLV reverse transcriptase. The expression profiles of the various PR genes were assessed via RT-PCR amplification of each of the cDNA fragments using the primers provided in Table 1.

Results

Generation of *swpa4*-overexpressing transgenic tobacco plants

Transgenic tobacco plants overexpressing the *swpa4* gene under the control of the CaMV 35S promoter were generated (Fig. 1a). Five independent transgenic lines were established and grown in a greenhouse to set seeds (T_1 seeds). The integration and gene copy numbers of the constructs in the transformed plants were analyzed via PCR analysis with *swpa4* and NPTII gene-specific primers (data not shown), then analyzed further via Southern-blot analysis (data not shown). Among the five transgenic lines, three lines 2, 3, and 4 showed a single copy insertion, whereas lines 1 and 5 exhibited multi-copy insertions. In the T_1 plant lines, Northern-blot analysis was conducted with the full-length *swpa4* cDNA as a probe (Fig. 1b). The expression of *swpa4* was detected in the leaves of all of the lines, but not in the control (empty vector) line, and a particularly prominent *swpa4* expression was detected in the leaves of

Table 1 RT-PCR primers of *swpa4* and various pathogenesis-related (PR) genes in tobacco plants

cDNA	Forward primer	Reverse primer	Accession No.
swpa4	CGGATCCATGGCTTCCTTTGT CACTCG	GGGATCCTCACATGGATGCAA CTG	AY206409
Acidic *PR1a*	AATATCCCACTCTTGCCG	CCTGGAGGATCATAGTTG	X12485
Acidic *PR1b*	ATCTCACTCTTCTCATGC	TACCTGGAGGATCATAGT	X12486
Acidic *PR2*	ACCATCAGACCAAGATGT	TGGCTAAGAGTGGAAGGT	DQ206348
Basic *PR2*	CAGCCCTGTCACTGGCACAT	CCCTACAGATGCCCCTCCTG	M59442
Acidic *PR3* (*PR-P*)	GGTAGTTGGACTCCGTCCGC	TCGTTTTCACTCGAAGCACCA	M29869
Acidic *PR3* (*PR-Q*)	ATCGGTCGTTGGACTCCGTC	GCCTCATCACTTAAAAGCGCC	M29868
Basic *PR3*	ATGAGGCTTTGTAAATTC	AGTCGCCGGGGCTACCTTC	S44869
Acidic *PR4*	TGCTTTCTGCGCTACTTGGG	GCCCCTTCTTATTCTAAACGGC	X58546
Basic *PR4*	ATGGTTGGAACTTCCGGA	TCCTGATCTCTCTGCTAC	AF154635
Basic *PR5*	ATGAGAAAGACCCACGTC	ATGCCTTCTTTGCAGCAG	AF154636
Acidic *PR9* (*NtPrx9*)	TGCACACACATTTGGAAGAGCA	TTGGAATTGCCATTTCAGCACA	J02979
Basic *PR14* (*LTP1*)	TGGTGTGCATGGCAGCAGTA	TCCATCTTCTCCAAGAACGCA	AY562132
Actin	TGGACTCTGGTGATGGTGTC	CCTCCAATCCAAACACTGTA	U60489

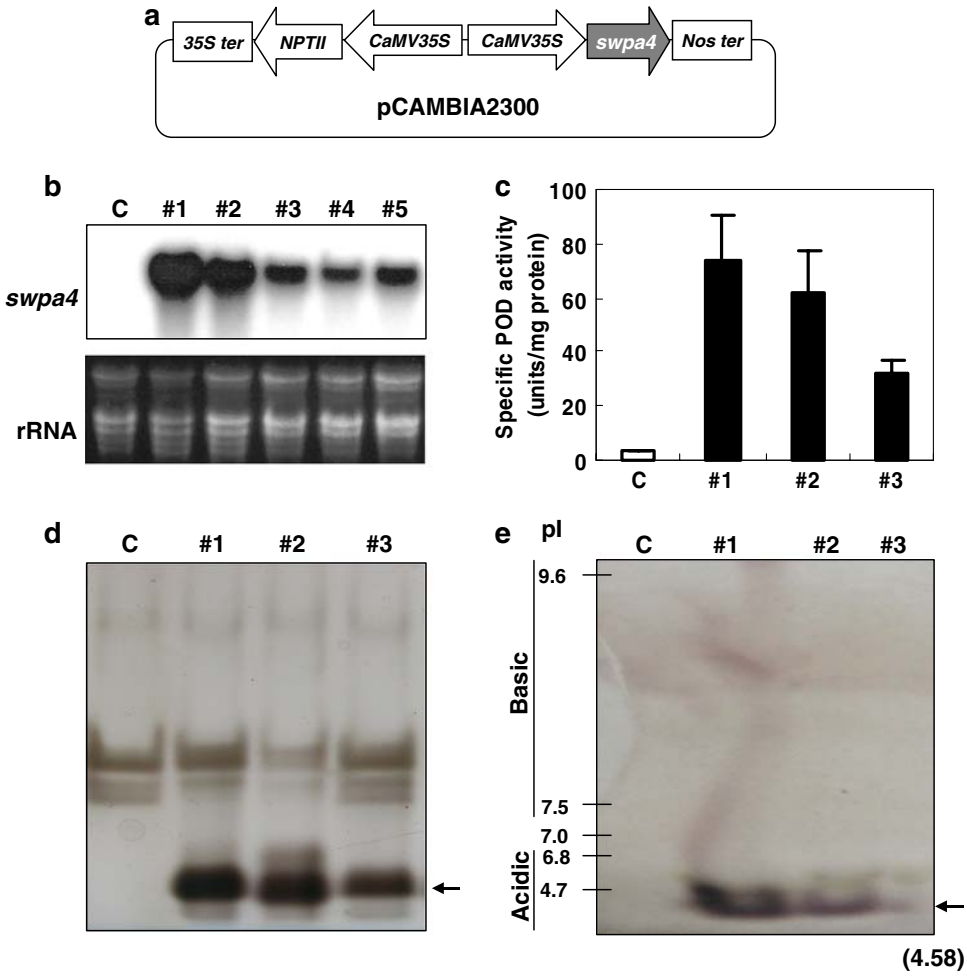

Fig. 1 Molecular and biochemical characterization of *swpa4* transgenic plants. **a** Diagram of constitutive expression of the 35S promoter::*swpa4* construct. **b** Northern-blot analysis of independent transgenic tobacco plants. **c** Specific POD activity in the *swpa4* transgenic plants. POD activity of the soluble extracts from leaves of 8-week-old tobacco plants was assessed using H_2O_2 and pyrogallol as substrate. Data represent the average of three replicates. **d** POD isoenzyme patterns from soluble leaf extracts by native PAGE **e** POD isoenzyme patterns from soluble leaf extracts by IEF on native gel. POD activity staining was conducted using H_2O_2 and benzidine as substrates. The *arrow* indicates the swpa4 POD enzyme produced by *swpa4* transgene expression under the control of the 35S promoter

transgenic lines 1 and 2. In order to ascertain whether *swpa4* expression was correlated with POD enzyme activity, we measured the POD activity in the soluble extracts from the leaves of the transgenic lines. The *swpa4* transgenic plants exhibited 20–50-fold higher POD activity than was observed in the control plants (Fig. 1c). Line 1 plants showed the highest levels of POD activity (73.65 units mg protein^{-1}). Activity staining of POD by native PAGE consistently revealed that all transgenic lines exhibited increased POD activity as compared to the control plants (Fig. 1d). We verified the acidic POD activity with approximately 4.5 of pI via IEF gel analysis only in the *swpa4* transgenic plants (Fig. 1e). This confirmed the occurrence of active swpa4 POD, as the predicted pI of swpa4 protein is 4.6. Although little POD activity was observed in the leaves of control plants (Fig. 1c), POD activity was detected by activity staining on gels (Fig. 1d). It appears that the different POD activity observed in both the enzyme assay and the gel activity staining are attributable to the specificity of the substrates, such as pyrogallol and benzidine, as benzidine indicates greater H_2O_2 oxidation capacity than pyrogallol (Hu and Kulkarni 2000). In the T_3 plants, three independent transgenic lines were selected for further analysis. The T_3 transgenic plants exhibited different phenotypes during growth under greenhouse conditions for 4 months (data not shown). The shoot growth of transgenic plants from line 1 and 2 was reduced by approximately 18% compared with control plants, whereas the shoot growth of line 3 plants was similar to that of controls.

Expression of swpa4 into the apoplastic space

The swpa4 protein was predicted to be secreted into the extracellular space according to the results of analyses with the PSORT program (http://psort.Ims.u-tokyo.ac.jp/form.html). In order to determine whether the swpa4 protein was targeted to the outside of cells, we performed an in-vivo targeting experiment using a swpa4::GFP construct (Fig. 2a). The fluorescence signal of the swpa4::GFP fusion was clearly located in the extracellular space. The extracellular localization was clarified in the presence of 10% sucrose, which induces plasmolysis, separating the cytosol from the cell wall and thus allowing the two compartments to be distinguished from one another. However, the fluores-

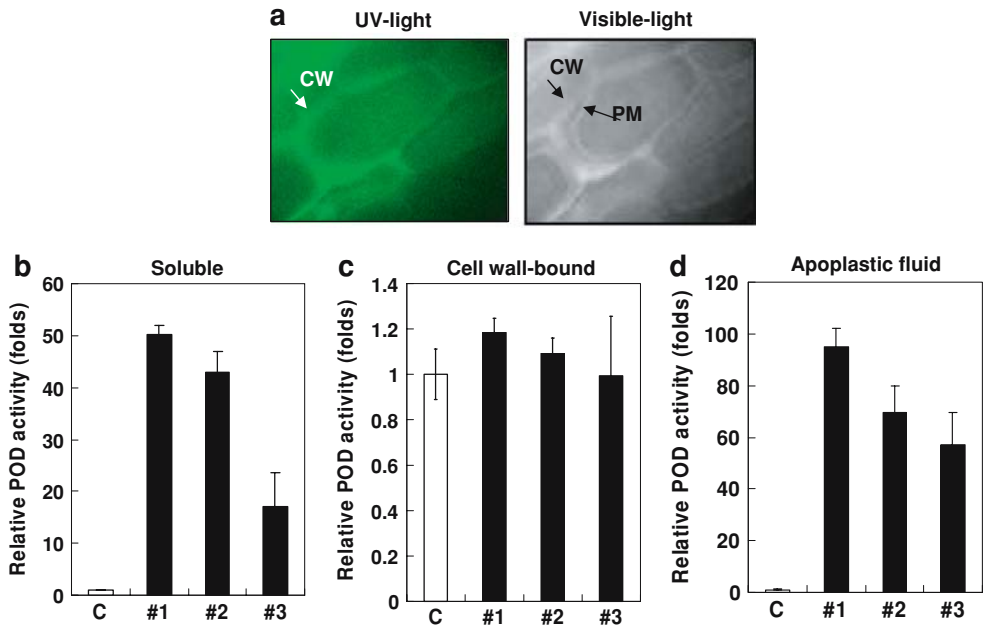

Fig. 2 Localization of the swpa4 protein into the apoplastic space. **a** Subcellular localization of swpa4. GFP fusion constructs were introduced into onion epidermal cells via particle bombardment. swpa4-GFP expression under plasmolysis by 10% sucrose was observed. *CW* cell wall, *PM* plasma membrane. **b** The POD activity in the soluble extracts from tobacco leaves. **c** The POD activity in the cell wall-bound extracts from tobacco leaves. **d** The POD activity in the apoplastic washing fluid from tobacco leaves. Data represent the average of five replicates

cence signal of the 35S*::GFP* control was predominantly detected within the cytosol (data not shown). These results indicate that expressed swpa4 was secreted into the extracellular space, including the cell wall.

In order to further assess the localization of swpa4 in the extracellular space, we evaluated the activity of POD in different sample fractions, including the soluble, cell wall-bound, and apoplastic washing fluid fractions. The transgenic plants showed an approximate 50-fold higher POD specific activity than was observed in the control plants in the soluble fraction (Fig. 2b). Cell wall-bound POD activity had a similar level in both the control and transgenic plants (Fig. 2c). Interestingly, specific activity of POD in the apoplastic fluid fraction of transgenic plants was approximately 100-fold higher than that of control plants, as shown by spectrophotometric analysis (Fig. 2d). In order to assess the levels of cytosolic contamination in the cell wall-bound or apoplastic washing fluid fractions, catalase activity was analyzed in each fraction, as catalase was used as a cytosolic molecular marker for leakage from broken cells (Polle et al. 1994). Catalase activity was detected in the soluble protein extracts, but not in the cell wall-bound and apoplastic fluid fractions (data not shown). Collectively, these results indicate that the swpa4 protein was secreted into the apoplastic space and was responsible for the observed enhanced POD activity. Therefore, we conclude that sweetpotato *swpa4* encodes for a functional acidic POD enzyme, which is expressed in the apoplastic space.

Swpa4 transgenic plants show tolerance to various abiotic stresses

It has been previously determined that elevated POD activity in plants is correlated with increased tolerance to a variety of abiotic stresses, including oxidative and osmotic stresses (Amaya et al. 1999; Yun et al. 2000). Therefore, we attempted to determine whether the *swpa4* transgenic plants show increased tolerance to oxidative stress. Here, we used three homozygous T_3 lines for further experiments. When the tobacco leaf discs were subjected to 2 μM MV, transgenic lines 1 and 2 showed approximately 26 and 22% reductions in membrane damage at 24 h, respectively, as compared with the control plants (Fig. 3a, c). After treatment with 200 mM H_2O_2, membrane damage of transgenic lines 1 and 2 was reduced for approximately 18 and 14% at 60 h, respectively, as compared with the control plants (Fig. 3b, d). Transgenic line 3 exhibited a similar level of membrane damage as control plants after treatment with MV or H_2O_2. The analysis of total Chl contents in the transgenic plants subjected to MV and H_2O_2 treatment revealed that transgenic lines 1 and 2, which exhibited the highest POD activity (Fig. 1c, d), also retained the highest levels of Chl content after MV or H_2O_2 treatment (Fig. 3e, f).

In order to assess the effects of *swpa4* expression on drought stress tolerance in soil-grown whole plants, 2-month-old plants were not watered for 8 days, and then watered for 8 days for recovery. More bleaching and a

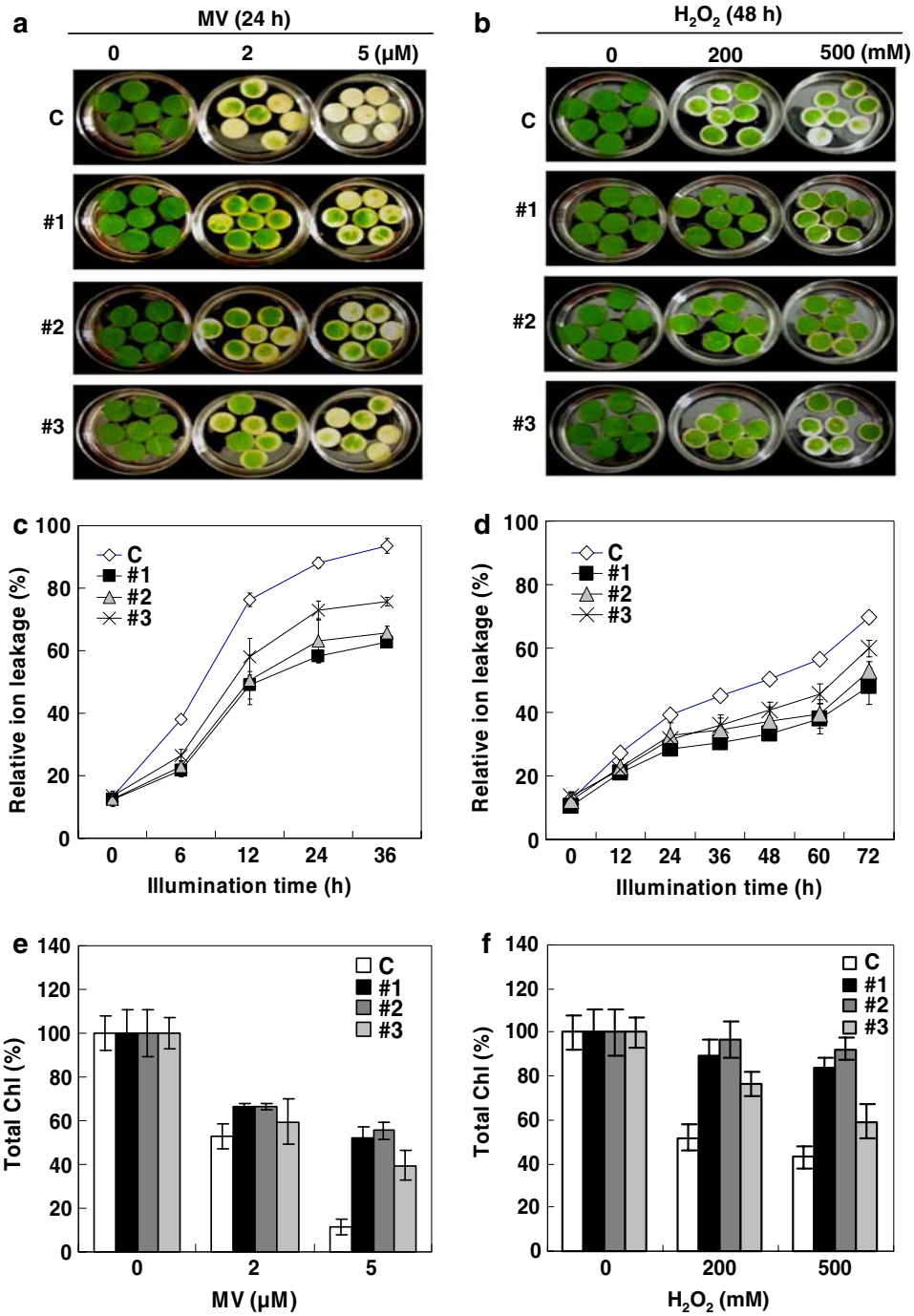

Fig. 3 Enhanced oxidative stress tolerance in the *swpa4* transgenic plants. **a** Photograph of leaf discs in the transgenic plants 24 h after MV treatment. **b** Photograph of leaf discs in the transgenic plants 48 h after H_2O_2 treatment. **c** Analysis of cellular damage in the MV-treated leaf discs of transgenic plants by electrolyte leakage for 36 h. **d** Analysis of cellular damage in H_2O_2-treated leaf discs of *swpa4* plants for 72 h. **e** Total Chl contents at 24 h after MV treatment. **f** Total Chl contents at 48 h after H_2O_2 treatment. Data represent the average of three replicates, each from seven leaf discs

greater loss of PSII photosynthetic efficiency was observed in the control plants compared to the transgenic plants (Fig. 4a). In the salt tolerance experiments (Fig. 4b), the *swpa4* transgenic lines recovered significantly better than the control plants with regard to Chl content. In addition, the tobacco leaf discs from transgenic lines 1 and 2 exhibited higher levels of tolerance in the presence of mannitol and NaCl , as seen by assessments of lipid peroxidation and total Chl (Fig. 4c, d). On the basis of the above results, it was evident that the *swpa4* transgenic plants are more tolerant to MV, H_2O_2, dehydration, and high salinity than the control plants.

Swpa4 transgenic plants show resistance to pathogens

POD is known to be activated in response to pathogenic attacks, and several functions in host-pathogen interactions have been attributed to plant POD (Hiraga et al. 2001; Passardi et al. 2005). In order to determine whether *swpa4* expression is involved in disease resistance, leaves of

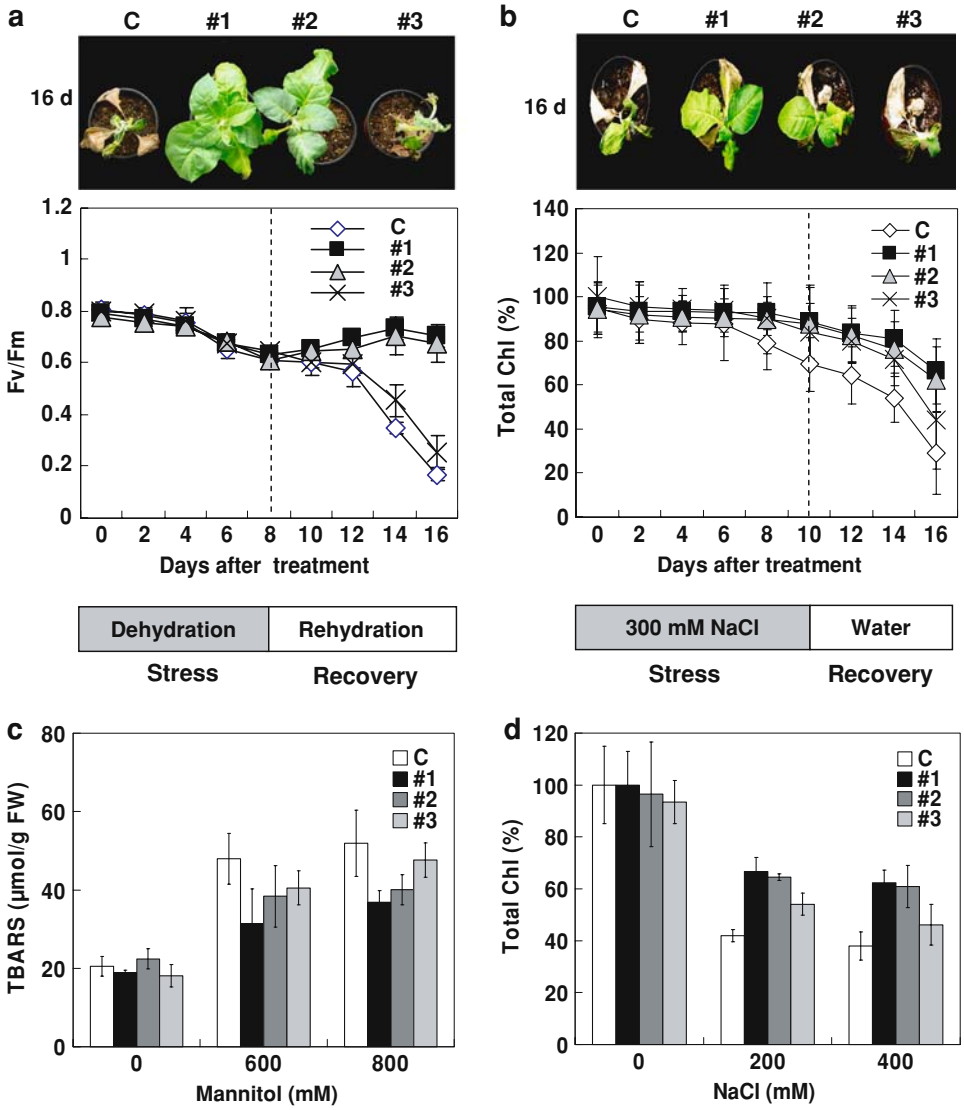

Fig. 4 Enhanced drought and salt stress tolerance in *swpa4* transgenic plants. **a** Drought stress tolerance. PSII photosynthetic efficiency in dehydration-treated transgenic plants was analyzed with a chlorophyll fluorescence meter. Fv/Fm is relative chlorophyll fluorescence showing PSII photosynthetic efficiency. F_v is the total amount of variable fluorescence and F_m is the maximum fluorescence yield. **b** Salt stress tolerance. Total Chl contents in salt-treated transgenic plants were analyzed. Data represent the average of three replicates, each from eight plants. **c** Lipid peroxidation analysis after mannitol treatment. **d** Total Chl contents after NaCl treatment. Data represent the average of three replicates, each from seven leaf discs

tobacco plants were inoculated with the fungal pathogen *P. parasitica* var *nicotianae* (Fig. 5a). The disease symptoms developed very quickly on the leaves of control plants over 3 days. However, no observable symptoms were detected on leaves of transgenic lines 1 and 2, and symptoms were significantly delayed in the line 3 plants. Cell death lesions as the result of fungal pathogenic infections were assessed via trypan blue staining (Fig. 5b). The data indicate that the overexpression of the *swpa4* gene in tobacco plants enhances disease resistance to fungal pathogens.

POD expression has been shown to confer an increased capability for insect resistance, via an induced increase in the generation of toxic metabolites (Dowd et al. 1998). Thus, we also assessed the effects of *swpa4* expression on insect resistance (Fig. 5c). The majority of the newly hatched *S. litura* larvae failed to survive in their first instar when bred with the *swpa4* transgenic plants, whereas a normal survival rate (≥80%) was observed with the control plants (data not shown). In transgenic lines 1 and 2, also a high degree of resistance to *S. litura* larvae feeding in the second instar was found (Fig. 5c), with only local lesions, and the larvae died within 4 days after feeding. By the third or fourth instar feeding, no significant differences in larval growth and survival rate were observed between the control and the transgenic plants (data not shown). These results inicate that *swpa4* expression in tobacco plants enhances resistance against insect feeding in the early developmental stages.

Expression of *swpa4* show increased lignin and phenolic content

High levels of POD activity increase phenolic and lignin contents (Lagrimini 1991). We thus studied POD activity, phenolic content, and lignified tissues in the stems of tobacco plants, via histochemical staining (Fig. 6a). The

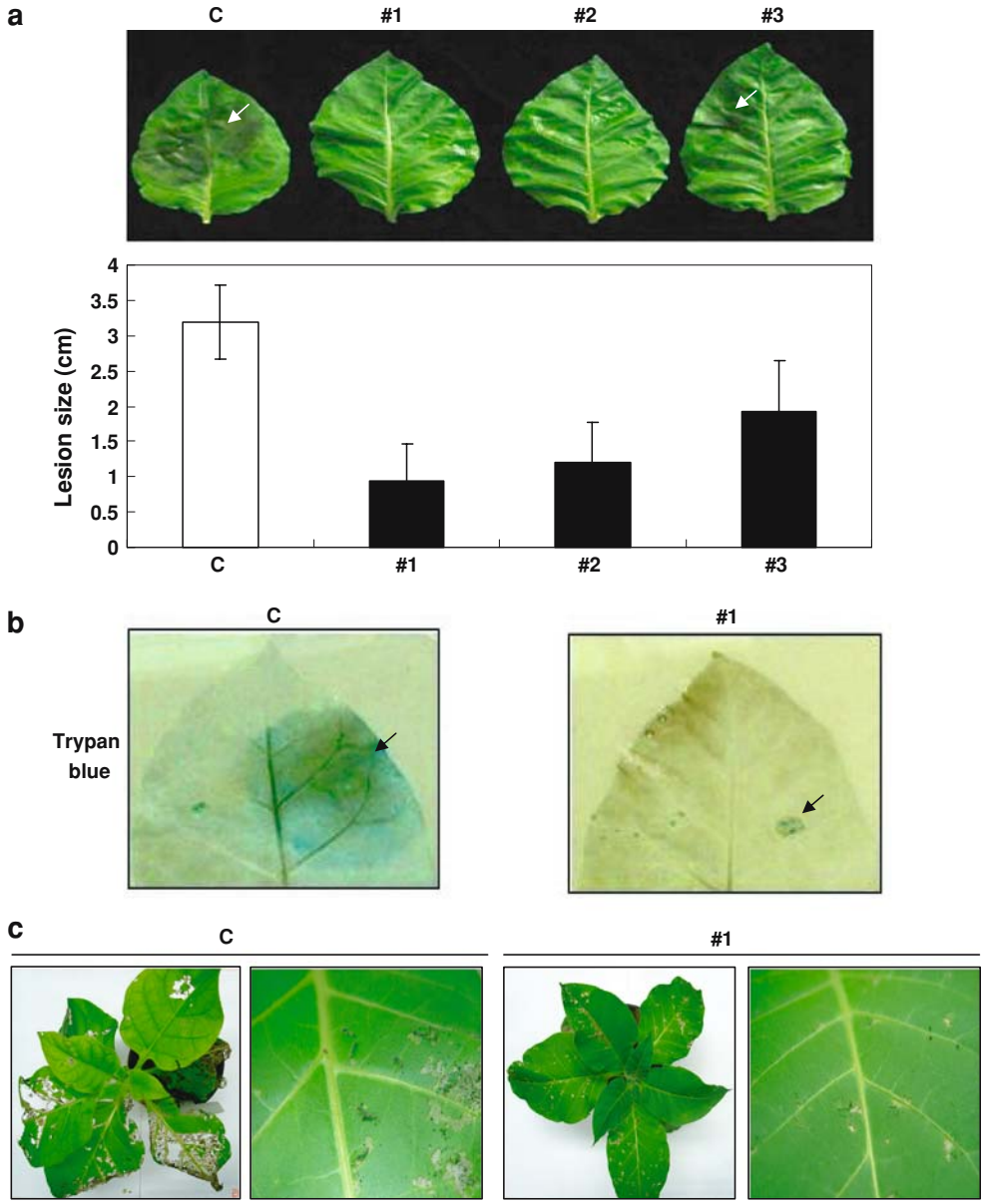

Fig. 5 Enhanced pathogen resistance in the *swpa4* transgenic plants. **a** Resistance to fungal pathogen *P. parasitica nicotianae*. The disease rating was scored according to the lesion size at 3 days post-inoculation as an average of the inoculated leaves of transgenic plants. The experiment was repeated three times. **b** Trypan blue staining of cell death. The leaves were stained with trypan blue 3 days after fungal pathogen inoculation to reveal dead cells. **c** Effects of insect feeding in the transgenic plants. A total of 50 second-instar lavae of *S. litura* were placed on the leaves of the transgenic and the control plants and were permitted to feed for 4 days

swpa4 transgenic plants had higher levels of POD activity in the xylem of the stem tissues than the control plants. With regard to phenolic content, the xylem of transgenic plant lines 1 and 2 was dark-green in color, whereas the xylem of line 3 plants stained in a fashion similar to that of the control plants. Lignin staining via phloroglucinol-HCl in the xylem of the plants indicated the presence of cinnamyl aldehyde groups in the cell walls. A dark red color was detected in the xylem of transgenic lines 1 and 2, which was indicative of increased lignin contents.

Via quantitative analysis, we determined the content of phenolics and lignin in the leaves of tobacco plants. Methanol extractable phenols in the transgenic plants were assayed in terms of *p*-coumaric acid contents (Fig. 6b). Contents of soluble phenolics from plant lines 1 and 2 were 1.9 and 2.2-fold higher than those detected in control plants, respectively. The lignin content of the transgenic plants was more than 1.5–2.2-fold higher than the one observed in the control plants (Fig. 6c). Qualitative and quantitative phenolic analyses were conducted with the

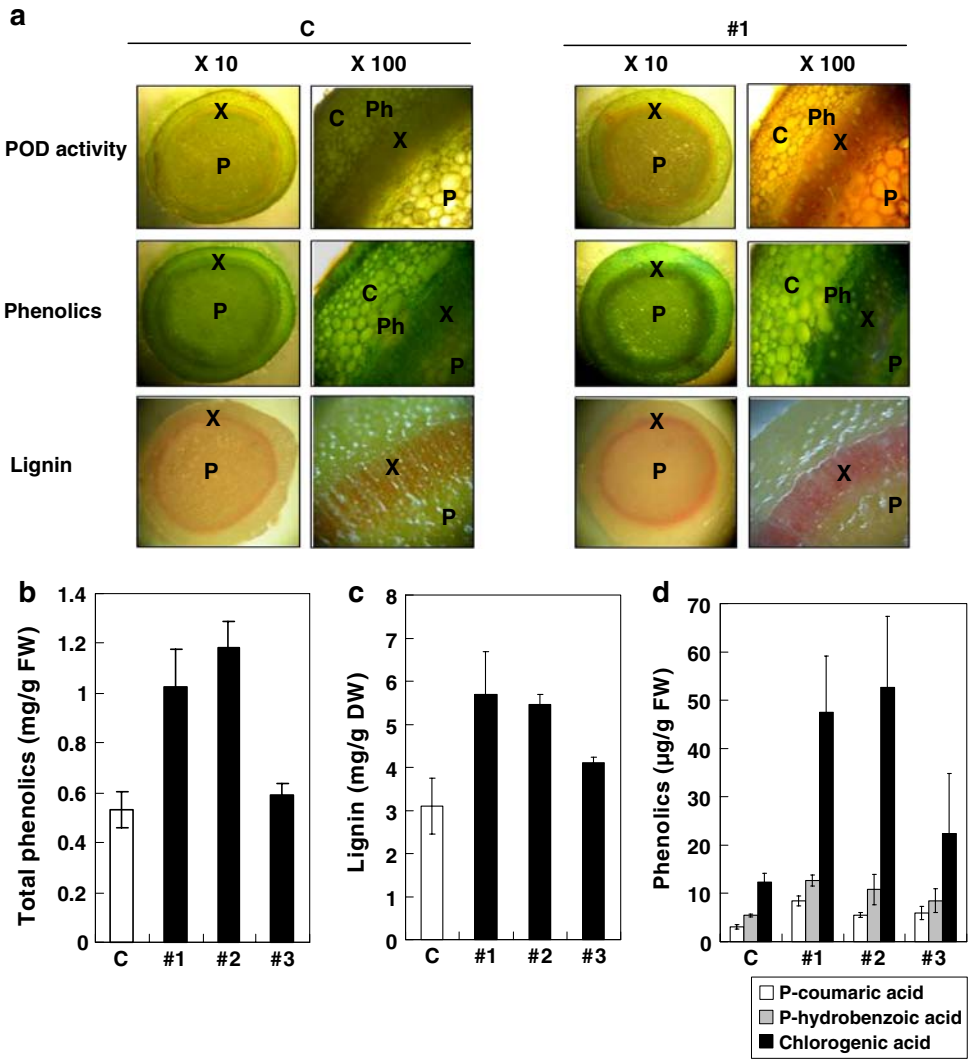

Fig. 6 Increased phenolics and lignin levels in the *swpa4* transgenic plants. **a** Histochemical analysis of the stem in transverse sections of the transgenic plants. POD activity staining (*top*). Phenolic staining of ferric chloride and potassium ferricyanide (*middle*). Phloroglucinol-HCl staining of lignified tissues (*bottom*). *C* cortex, *ph* phloem, *X* xylem, *P* pith. **b** Soluble phenolic contents in the leaves. **c** Lignin contents in the leaves. **d** HPLC analysis of representative phenolics. Data represent the average of five replicates

transgenic plants via HPLC analysis. The representative phenolic acids of tobacco leaves were *p*-coumaric, *p*-hydroxybenzoic, and chlorogenic acids (Fig. 6d). They were all elevated in transgenic plants compared to control plants. In particular, the chlorogenic acid content was highest in the transgenic plants and was three to four-fold higher than in the controls. From the above data, we concluded that the increase of phenolic and lignin resulting from the expression of *swpa4* might be associated with defense responses under a variety of abiotic and biotic stresses.

Expression of *swpa4* induces H_2O_2 production in transgenic tobacco plants

H_2O_2 is an electron-accepting substrate for a wide variety of POD-dependent reactions, and POD is therefore generally considered to be a scavenging reactive oxygen species (ROS; Passardi et al. 2005). Extracellular POD can also generate ROS as a by-product of its reactions (Bolwell et al. 2002; Mika et al. 2004). We thus attempted to determine whether *swpa4* expression results in an increase in H_2O_2 production in *swpa4* transgenic plants (Fig. 7a). By DAB staining, higher levels of H_2O_2 were detected in the *swpa4* transgenic plants compared to controls. In particular, the accumulation of H_2O_2 was eight-fold higher in the leaves of transgenic lines 1 and 2 than in the leaves of control plants. In an attempt to further address the direct role of *swpa4* in H_2O_2 production, we assessed H_2O_2 levels in the *swpa4* transgenic plants in the presence of the POD inhibitors, NaN_3 and KCN (Ellis and Duford 1968; Bolwell 1995). As is shown in Fig. 7b, H_2O_2 content in *swpa4* transgenic lines 1 and 2 was reduced to approximately 56 and 45%, respectively, 12 h after pretreatment with NaN_3 and KCN. In control plants, however, the levels of H_2O_2 were only slightly reduced in the presence of NaN_3 and KCN. These results show that the expression of *swpa4* performs a crucial function in the production of H_2O_2 in the transgenic plants.

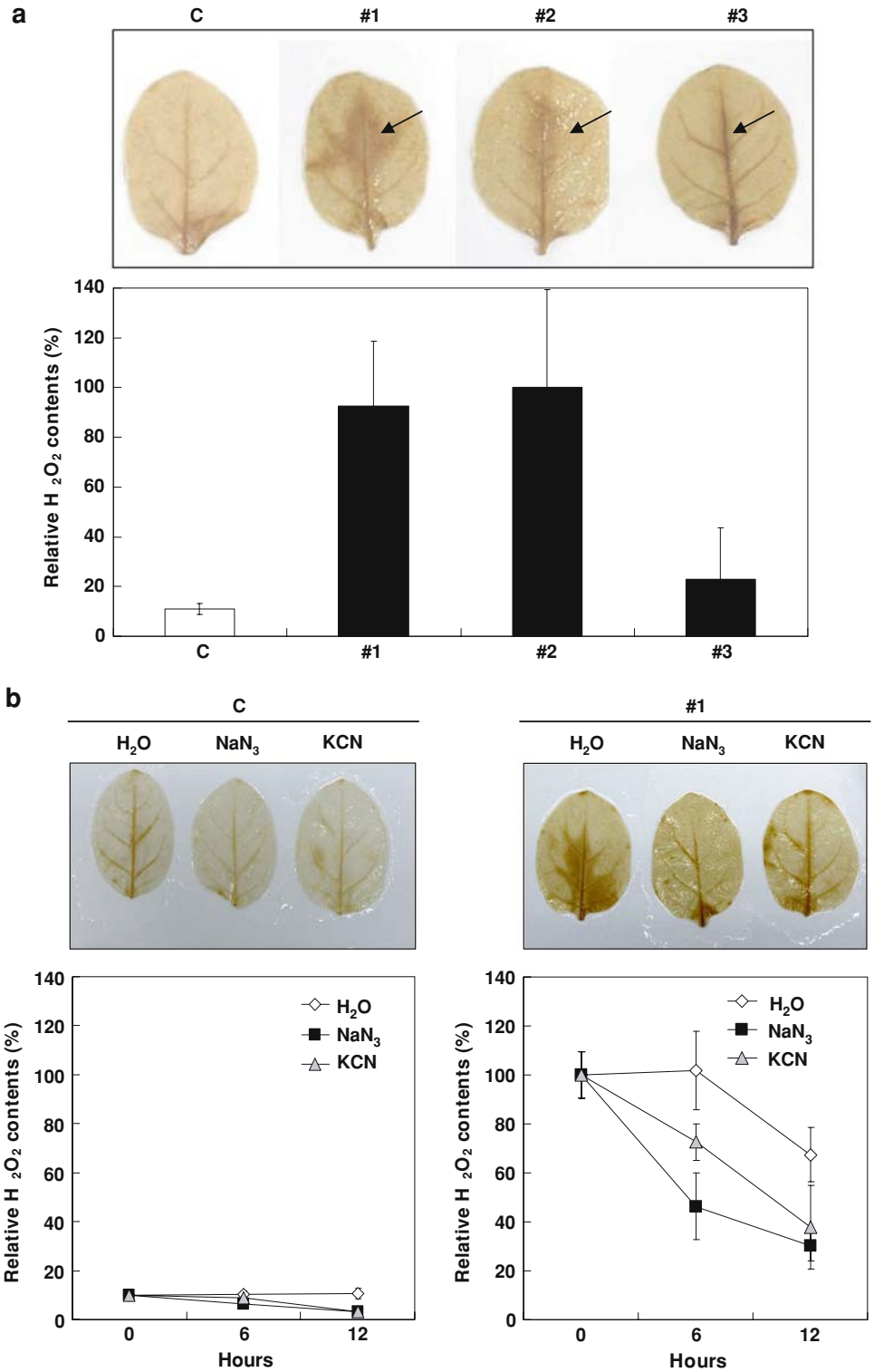

Fig. 7 Qualitative and quantitative analysis of H_2O_2 in the *swpa4* transgenic plants. **a** Visualization and determination of H_2O_2 contents in tobacco leaves. **b** Changes in H_2O_2 contents following treatment of POD inhibitor in tobacco leaves. Data represent the average of five replicates

The expression of *swpa4* up-regulates a variety of apoplastic PR genes via the regulation of H_2O_2

As an enhanced pathogenic resistance was found in the *swpa4* transgenic plants, we conducted RT-PCR in an effort to characterize the expression patterns of a variety of PR genes in the *swpa4* transgenic plants (Fig. 8a). The expression levels of extracellular acidic *PR2* (β-1,3-glucanase), *PR3* (*PR-P* and *PR-Q*, chitinase), *PR4* (chitinase), and *PR9* (lignin-forming POD, *NtPrx9*) genes were substantially increased in the *swpa4* transgenic plants. In an effort to find a correlation of *swpa4* expression with H_2O_2 generation

and the expression of defense genes, changes in transcripts were analyzed in the transgenic plants following treatment with POD inhibitors (Fig. 8b). As had been expected, the expression levels of extracellular *PR2*, *PR3*, *PR4*, and *PR9* were reduced following treatment with NaN_3 and KCN. Our consistent results strongly indicated that the generation of H_2O_2 by *swpa4* expression in the transgenic plants induced the expression of extracellular acidic PR genes. We conclude that increased H_2O_2 production as the result of *swpa4* expression is most likely related to the defense responses observed under various abiotic and biotic stress conditions.

Discussion

Over the entire life of a plant, POD performs important functions in plants, e.g., by controlling growth and development and defense responses (Passardi et al. 2005). Despite considerable efforts to investigate POD genes, their physiological functions as isoenzymes remain incompletely understood. In this study, we assessed the functions of *swpa4* isolated from the cell cultures of sweetpotato in transgenic tobacco plants. Interestingly, transgenic plants overexpressing *swpa4* in the apoplasts showed enhanced tolerance to a variety of abiotic and biotic stresses, thereby indicating that *swpa4* expression in the apoplastic space may function as a positive defense signal in the H_2O_2-regulated stress response signaling pathway.

The POD activity in the *swpa4* transgenic plant was approximately 50-fold higher than in control plants (Figs. 1c, 2b). Furthermore, the expressed swpa4 protein was secreted into the apoplastic space (Fig. 2). Apoplastic spaces, including the cell wall matrices, perform a prominent role in plant defense mechanisms. The involvement of the apoplastic spaces in these phenomena is associated with complex mechanisms, including changes in the synthesis of extracellular enzymes and other proteins (Marentes et al. 1993; Fecht-Christoffers et al. 2003; Dani et al. 2005). For example, tomato cell wall POD (*TPX2*) transgenic tobacco increased the germination rate under high salt and osmotic stress conditions (Amaya et al. 1999). Tobacco anionic POD (*TAP*) transgenic plants showed an enhanced resistance against insect attack (Dowd and Lagrimini 1997, 1998, 2006). Spruce cationic POD (*spi2*) transgenic tobacco also enhanced the resistance to pathogenic bacterial infections (Elfstrand et al. 2002). In particular, the expression of several apoplastic acidic PR genes was shown to have increased in salt-treated tobacco leaves (Dani et al. 2005). As expected, the *swpa4* transgenic plants showed a significantly enhanced tolerance to various abiotic and biotic stress conditions, with high levels of POD production occurring in the apoplast (Figs. 3, 4, 5). However, there appear to be differences in growth and stress-resistance phenotypes among the three transgenic lines. These differences are likely attributable to differences in POD activity. Even though the POD activity of the line 3 plants was increased by approximately 20-fold, the line 3 plants did not produce a significantly increased resistance phenotype (Fig. 1c). When considering the POD activity in

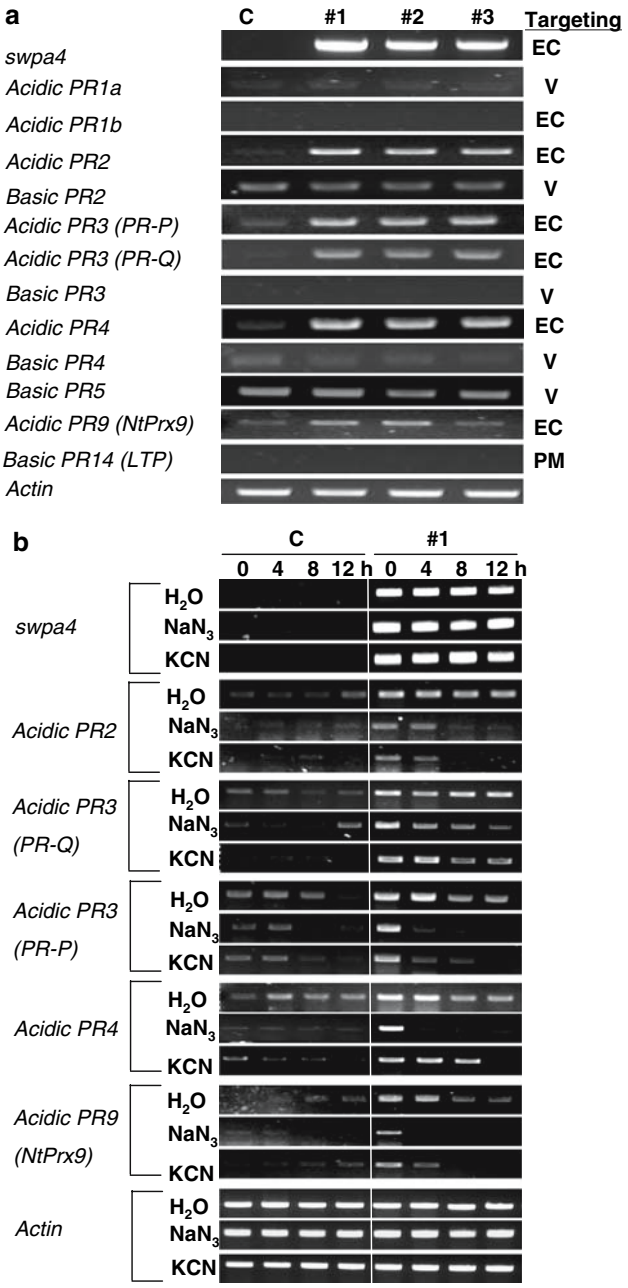

Fig. 8 Expression patterns of various PR genes in the *swpa4* transgenic plants. **a** RT-PCR analysis of various PR genes under normal conditions. *EC* extracellular, *PM* plasma membrane, *V* vacuole. **b** Reduced expression of acidic apoplastic PR genes after treatment with NaN_3 or KCN. Amplification of the *β-actin* gene is shown as a control for each sample

the apoplastic fluid, the difference between lines 2 and 3 became even less remarkable (Fig. 2d). It is possible that the lack of a resistance phenotype in the line 3 plants may be a consequence of certain positional effects during the tissue culture, and/or threshold effects of POD activity on resistance.

POD activity was found to result in an accumulation of various phenolics and lignin in plants (Passardi et al. 2004; Takahama 2004). Phenolics accumulate within the vacuole and apoplast in response to a variety of biotic and abiotic stresses. Takahama (2004) suggested a possible role of phenolics as signal molecules or in direct defense. Lignin is also responsible for the inhibition of expansion growth in plants and for the provision of physical barriers to a variety of stresses (Lagrimini 1991; Lee et al. 2007). In particular, chlorogenic acid has been reported to function as one of the major secondary metabolites in tobacco plants (Snook et al. 1986). Oxidized chlorogenic acid generates a toxic secondary metabolite guarding against insect attack in plants (Duffey and Stout 1996). Dowd et al. (1997, 1998, 2006) reported that the overexpression of tobacco anionic POD in transgenic plants induced enhanced resistance to insects due to a toxic metabolite generated from chlorogenic acid. Consistent with these results, we have demonstrated that the *swpa4* transgenic plants showed increased levels of lignin and phenolics, including chlorogenic acid, as well as enhanced resistance against biotic stresses (Figs. 5, 6). These reports and our results show that the overexpression of *swpa4* in tobacco plants may induce a physical and chemical defense response via lignification and phenolic accumulation. In particular, POD activity increased by *swpa4* expression may result in the enhanced generation of toxic metabolites from chlorogenic acid, and thus enhanced resistance against insect feeding.

H_2O_2 plays a dual role in plants. At low concentrations, it functions as a messenger molecule that is involved in signaling regulation, triggering tolerance against various abiotic and biotic stresses (Bolwell 1999; Desikan et al. 2001), and at high concentrations, it controls programmed cell death via oxidative burst (Mika et al. 2004; Bindschedler et al. 2006). Two major mechanisms, involving apoplastic POD and plasma membrane NADPH-oxidase, may explain ROS generation (Bolwell 1995). An increasing body of evidence also suggests that H_2O_2 is the key molecule controlling the activation of defense-related genes (Bolwell 1999; Desikan et al. 2001). For example, an enhanced expression of PR proteins in the absence of pathogenic challenge occurs in catalase suppressing transgenic plants with increased H_2O_2 production (Chamnongpol et al. 1998). Therefore, it was suggested that H_2O_2 may play a major role in the signaling cascade leading from pathogen infection to PR protein expression. H_2O_2 also functions as a substrate in stress-related biosynthetic pathways such as lignification, suberization, and protein -dependent cell wall reinforcement (Pomar et al. 2002). We demonstrated that the overexpression of *swpa4* induced H_2O_2 generation in transgenic plants (Fig. 7a). Such H_2O_2 levels were reduced by inhibitors of POD, including NaN_3 and KCN, which suggest that the expression of *swpa4* results in H_2O_2 production (Fig. 7b). It is likely that such H_2O_2 generation results from accumulation in the apoplastic space, as *swpa4* expression is localized in the apoplasts. We also determined that the expression of *swpa4* resulted in the induction of a variety of acidic PR genes, including *PR2*, *PR3*, *PR4*, and *PR9* (Fig. 8a). A majority of acidic PR proteins were secreted into the apoplasts, whereas basic PR proteins were located in the vacuoles (Van Loon et al. 2006). The extracellular PR genes were located in a manner conducive to their contact with invading attackers before tissue penetration occurs, and thus they were considered a possible first defense. However, when the pathogen succeeds in causing tissue damage, vacuolar PR genes might operate as an effective second defense mechanism as well (Van Loon et al. 2006). This corroborates the notion that accumulation of H_2O_2 as the result of *swpa4* expression in the apoplasts was correlated with primary defense mechanisms involving the induction of extracellular PR gene expression.

According to our results, *swpa4* expression exhibited a variety of functions in plant POD, including lignification, phenolic accumulation, H_2O_2 generation, and various stress resistance functions. It appears likely that the involvement of *swpa4* in these multiple functions may be the consequence of the regulation of increased H_2O_2 production. It is well known that H_2O_2 performs a crucial signal molecule function in many aspects of the signaling pathways. Therefore, we suggest that H_2O_2 generation by *swpa4* may be responsible for the multiple functions of POD.

Further investigation will be required in order to elucidate the exact role of *swpa4* in the regulation of H_2O_2 production in the apoplasts of sweetpotato under stress conditions. Recently, we developed transgenic sweetpotato plants under the control of a stress-inducible sweetpotato POD promoter (Kim et al. 2003; Lim et al. 2007). We expect that the overexpression or suppression of apoplastic POD in transgenic sweetpotato plants may provide us with valuable information regarding the development of crops tolerant to a variety of stresses. The multiple stress-inducible *SWPA4* promoter remains to be studied for biotechnical applications.

In summary, we demonstrate that the overexpression of apoplastic *swpa4* in transgenic tobacco plants showed tolerance to a variety of abiotic and biotic stresses. Phenolic compounds and lignin accumulation increased in the *swpa4* transgenic plants. Moreover, *swpa4* expression caused an increase in H_2O_2 production followed by the induction of defense-related genes, including the apoplastic acidic PR genes.

Acknowledgments This work was supported by grants from the Environmental Biotechnology National Core Research Center, KOSEF/MOST, from the Korea Foundation for International Cooperation of Science and Technology (KICOS), MOST and from Biogreen21 Program (20070301034015), Rural Development Administration, Korea.

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