Overexpression of a pepper ascorbate peroxidase-like 1 gene in tobacco plants enhances tolerance to oxidative stress and pathogens

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Abstract

In order to determine the role of ascorbate peroxidase, an antioxidant enzyme, in the cellular responses to oxidative stress and pathogens, transgenic tobacco plants were generated, using the *Capsicum annuum* ascorbate peroxidase-like 1 gene (*CAPOA*1), under the control of the CaMV 35S promoter. High levels of *CAPOA*1 gene expression were observed in the transgenic plants, with a 2-fold increase in total peroxidase activity. The constitutive expression of *CAPOA*1 in the tobacco exhibited no morphological abnormalities, while significantly increased growth was observed in transgenic plants, as compared to control plants. The *CAPOA*1-overexpressed plants exhibited increased tolerance to methyl viologen-mediated oxidative stress, and also enhanced resistance to the oomycete pathogen, *Phytophthora nicotianae*. However, the transgenic plants were not found to be resistant to the bacterial pathogen, *Pseudomonas syringae* pv. *tabaci*, but were weakly resistant to *Ralstonia solanacearum*. Our results suggested that the overproduction of ascorbate peroxidase increased peroxidase activity that enhances active oxygen scavenging system, leading to oxidative stress tolerance and oomycete pathogen resistance.
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1. Introduction

Plants often face the challenge of severe environmental conditions, which include such stressors as drought, salinity, high and low temperatures, UV radiation, and pathogenic attacks, all of which exert adverse effects on plant growth and development. Reactive oxygen species (ROS), such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH^-), are some of the most damaging stressors in plants, and occur particularly in high and low temperatures, in combination with high light intensity [1], drought, exposure to air pollutants (e.g. ozone or sulfur dioxide), UV light, and herbicides such as paraquat [2]. Even under normal conditions, higher plants often produce reactive oxygen species during metabolic processes, via the Mehler reaction in the chloroplasts, electron transport in the mitochondria, and photorespiration in the peroxisomes [3]. Environmental stresses can produce excess concentrations of ROS, resulting in oxidative damage to, or the apoptotic death of, cells.

Development of an antioxidant defense system in plants protects them against oxidative stress damage, by either the partial suppression of ROS production, or the scavenging of ROS which has already been produced [4]. Different non-enzymatic (ascorbate, glutathione, polyamines, α-tocopherol, and carotenoids) and enzymatic

(superoxide dismutase, ascorbate peroxidase, glutathione reductase, and catalase) molecules are involved in scavenging excess ROS in plant cells [5]. Peroxidase is a ubiquitous enzyme which catalyzes the oxidation of cellular components. In plants, peroxidases play several biological roles, such as lignin biosynthesis, indole-3-acetic acid degradation or ethylene biosynthesis, on the basis of their specific electron donor activities and the ability of scavenging hydrogen peroxide or organic hydroperoxides [6]. Ascorbate peroxidase (APX) is one of the most important key enzymes that scavenge potentially harmful H_2O_2 from the chloroplasts and cytosol of a plant cell [7]. It utilizes ascorbate as a specific electron donor, as ascorbate is a very important reducing substrate for H_2O_2 detoxification in the photosynthetic organism. It is important to scavenge H_2O_2 because this active oxygen molecule produces highly reactive hydroxyl radicals and these radicals oxidize almost all cell components, causing cellular damage [8]. It is also reported as an important defense enzyme, which protects plant cells from disease agents via systemic acquired resistance [9].

Increases in peroxidase level during plant-pathogen interactions are a well-documented phenomenon. In our previous study, increased endogenous peroxidase transcription was observed in pepper plants infected with *Xanthomonas campestris* pv. *vesicatoria* [10]. Up-regulated expression of the ascorbate peroxidase gene was also observed in barley leaves, after inoculation with *Blumeria graminis* f.sp. *hordei* [11].

There are four APX isoforms, which can be isolated from four distinct cellular compartments; stroma APX (sAPX) [12], thylakoid membrane-bound APX (tAPX) [13], microbody membrane-bound APX (mAPX), and cytosolic APX (cAPX) [14]. More recently, a fifth APX isoenzyme has been identified in the mitochondrial membranes (mitAPX) [15,16]. The precise defensive roles played by each isoenzyme have not yet been established. However, it is believed that the peroxidases are involved in some physiological processes, such as the strengthening of the cell wall via lignin deposition [17] or the cross-linking of cell wall proteins [18]. These phenomena might constitute the general defense mechanism of plants against a broad range of pathogens.

During the last few years, considerable progress has been made in understanding how plants protect themselves against oxidative stresses and pathogens. Several genes encoding for plant antioxidant enzymes have been cloned, characterized, and used in the construction of transgenic lines. The overproduction of superoxide dismutases [19] and glutathione reductase [20] has been shown to confer increased tolerance to oxidative stresses. Pathogenesis-related peroxidase (*Shpx*2 and *Shpx*6a) has also been reported to enhance resistance against oomycete pathogens [21,22].

In this study, in an attempt to identify the potential functions of the ascorbate peroxidase-like 1 gene, we transformed tobacco plants using a pepper CAPOA1 cDNA

(NCBI accession no. AF442387), which encodes 287 amino acids and is 66% identical to tobacco, tomato, bell pepper and 67% to cucumber [10]. Here, we report that the constitutive expression of the pepper *CAPOA*1 gene in transgenic plants enhanced cellular protection against MV-mediated damage, and conferred resistance against the oomycete pathogen, *Phytophthora nicotianae*, but not against the bacterial pathogens, *Ralstonia solanacearum* and *Pseudomonas syringae* pv. *tabaci*.

2. Materials and methods

2.1. Construction of plant expression vector and Agrobacterium transformation

The pepper gene coding for ascorbate peroxidase-like 1 (*CAPOA*1), which we have previously cloned [10], was used in this study. The plasmid pBluescript SK(−) harboring the DNA sequences coding for *CAPOA*1 was amplified with two gene-specific primers: *CAPOA*1-5(5′-GGG<u>GGATCC</u>G-CACGAGGTCGGTTCTCTCTC-3′) and *CAPOA*1-3 (5′-TTT<u>GAGCTC</u>CCTCCAAAGTATGGGTATC-3′). This amplified product was digested by *Bam*HI and *Sac*1 (underlined positions of these primers) and ligated into the corresponding site of the plant expression vector, pMBP1 [23]. The resulting recombinant plasmid, pMBP1-*CAPOA*1, containing the 35S cauliflower mosaic virus promoter, nopaline synthase terminator, neomycin phosphotransferase, and the kanamycin resistance marker gene (Fig. 1A), was transformed into *A. tumefaciens* strain EHA 105.

2.2. Tobacco transformation and T_1 seed production

Six- to eight-week-old *Nicotiana tabacum* cv. xanthi plants were used to generate transgenic plants, utilizing the pMBP1-*CAPOA*1 construct. *A. tumefaciens*-mediated tobacco leaf disc transformation was carried out according to the procedure described in [24]. Briefly, leaf discs were co-cultured with *Agrobacterium* using liquid MS [25] medium for 3 days at 28 °C in the dark, followed by washing with liquid MS medium containing 250 mg/l carbenicillin and with sterilize water for 2 to 3 times. Leaf discs were cultured on the regenerating medium (MS + 2 mg/l benzylaminopurine (BA) + 0.1 mg/l naphthaleneacetic acid (NAA) + 30 g/l sucrose + 100 mg/l kanamycin + 250 mg/l carbenicillin). Healthy resistant shoots were then transferred to a rooting medium (MS + 1 mg/l 3-indolebutyric acid (IBA) + 30 g/l sucrose + 150 mg/l kanamycin). All the cultures were kept in the growth chamber at 24–26 °C under a 16 h photoperiod provided by cool-white fluorescent lamps (40 μmol m^{-2} s^{-1}). Four well-developed rooted plants were transferred to soil, and then grown in a greenhouse. T_1 seeds were collected via self-pollination, and used for further experiments. Empty vector (pMBP1)-transformed plants were used as a control.
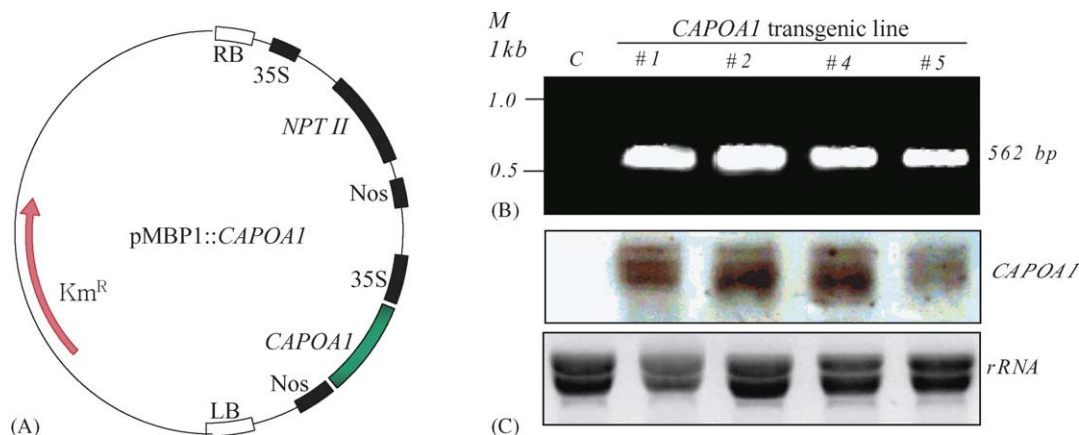
Fig. 1. Generation of the *CAPOA*1 transgenic tobacco plants. (A) Diagram of the constructed plant expression pMBP1-*CAPOA*1 vector. RB, right border; LB, left border; *NPT*II, neomycin phosphotransferase; 35S, cauliflower mosaic virus 35S promoter; *NOS*, nopaline synthase terminator. (B) Amplification of *CAPOA*1 from the genomic DNAs of four different transgenic plants. M, marker; C, control plants (pMBP1-transformed plants); transgenic lines, #1, #2, #4, and #5. (C) Northern blot analysis. C, control plants (pMBP1-transformed plants); transgenic lines, #1, #2, #4, and #5.

2.3. Genomic DNA isolation and PCR screening for transgenic plants

Genomic DNA was isolated according to the procedure described in [26]. Leaves were punched with a 1.5 ml microcentrifuge tube lid, and then homogenized with a pellet pestle in 400 μl of genomic DNA extraction buffer (200 mM Tris–HCl, pH 7.5; 250 mM NaCl; 25 mM EDTA; 0.5% SDS). PCR was carried out with the two gene-specific primers: *CAPOA*1-5 and *CAPOA*1-3. Amplification was performed under the following conditions: 95 °C for 3 min, 30 cycles of 95 °C for 20 s, 58 °C for 30 s, and 72 °C for 1 min. Finally, the PCR products were separated onto a 1% agarose gel, and visualized by ethidium bromide staining.

2.4. RNA isolation and Northern blot analysis

Total RNAs were prepared using TRIzol® Reagent (Gibco-BRL, USA), according to the manufacturer's instructions. Northern blot analysis was carried out using the Southern-*Star*™ detection kit (Tropix, USA) for biotin-labeled DNA. An amount of 10 μg of total RNAs were fractionated on a 1% denaturing agarose gel (1×MOPS buffer, 15% formaldehyde, 1% agarose), transferred onto a Tropilon-plus™ membrane (Tropix, USA) by the alkaline transfer method, and then cross-linked with UV irradiation. Hybridization was carried out using a biotin-labeled *CAPOA*1 probe, at 68 °C for 16 h.

2.5. Total peroxidase activity assay

Peroxidase activity was analyzed according to the method described in [10]. An amount of 1 g of leaf tissue was ground in liquid nitrogen, and then homogenized in 10 volumes of an extraction buffer (10 mM sodium phosphate and 1% sodium metabisulphite, pH 6.0) per unit fresh weight. After centrifugation at $13,000 \times g$ for 30 min at 4 °C, the supernatant was stored at −70 °C. The total

peroxidase activity of the crude extracts was spectro-photometrically measured at 470 nm, and guaiacol was used as a hydrogen donor. The reaction mixture was composed of 2.9 ml phosphate buffer (10 mM, pH 6.0, 25 °C), with 1.25% (v/v) guaiacol, and 0.1 M H_2O_2. The enzyme reaction commenced upon the addition of 100 μl of enzyme extract, and the absorbance was recorded at 470 nm, at 25 °C for 5 min. Crude enzyme extracts were diluted in order to produce changes in absorbance, at 470 nm, of 0.1–0.2 absorbance units per min. The peroxidase activity of the crude extracts was calculated using the molar extinction coefficient of tetraguaiacol (26.6×10^3 mol/cm), and was expressed as nano katals per milligram of total protein.

2.6. Bioassay of oxidative stress tolerance

In order to assess oxidative stress tolerance in the transgenic plants, methyl viologen, a superoxide-generating compound, was applied to both the control and T_1 plants. Methyl viologen (MV) dissolved in Tween 20 (0.1%) was used at 50 and/or 100 μM for each treatment. Three independent experiments were performed for oxidative stress tolerance. Leaf discs (1 cm²) of control and transgenic lines were treated with MV (50 and 100 μM) and kept in the plant growth chamber with continuous light at 28 °C for 9 h. Nine leaf discs, from nine plants of each line, were used in this experiment. Chlorophyll content was determined 9 h after treatment (Fig. 4A). The T_1 seeds of the control and the two lines were germinated on a MS medium [25] containing kanamycin 50 mg/l. A total of 35 seedlings (10 days old) were transferred to the MS medium containing 50 μM MV. Seedlings were transferred to a MS basal medium 4 days after treatment, for purposes of recovery. The tolerance levels of the transgenic plants to MV-mediated oxidative stress were assessed at the whole plant level. Both young seedling plants (5 weeks old) and mature plants (8–10 weeks old) of the control and the four transgenic lines were used for this purpose. An amount of 20 ml of MV (50 μM) was

sprayed on 6 plants (5 weeks old) of each line with a total of 12 plants, and 20 ml was sprayed on two mature plants of each line. All plants used in this experiment were grown in 10 cm × 12 cm pots. Photos were taken 4 days after treatment.

2.7. Pathogen inoculation procedures

The oomycete pathogen *P. nicotianae,* which causes Black Shank disease, was grown in darkness, at 26 °C on V8 juice agar medium (200 ml V8 juice, 15 g Bactoagar, 3 g $CaCO_3$ in 1 l dH_2O). For the detached-leaf assay, fully expanded leaves of 5- or 6-week-old plants were used for oomycete inoculation. An oomycete plug of 5-day-old culture (0.5 mm in diameter) was placed at the center of the adaxial leaf surface. The inoculated leaves were then kept on two layers of moist filter paper in a Petri dish, under a 16 h photoperiod, at 25 °C. A total of seven leaves from seven plants of each line were used for oomycete infection, and this experiment was replicated at least twice. The infected leaf area was measured 5 days after inoculation with *P. nicotianae*. In order to infect the stems, 0.5 to 1 cm^2 of bark from the center of the stems was removed with a sharp sterilized blade, and a mycelium plug was placed onto the wounded site. At least three replications were performed, with six to eight plants of each line per replicate. The inoculated plants were kept in a growth chamber, at 100% humidity and a 16 h photoperiod, at 25 °C. Photos were taken 30 days after infection.

2.8. Subcellular localization of CAPOA1 in onion epidermal cells

The coding sequence of the *CAPOA1* gene was amplified, using gene-specific primers of *CAPOA1*-5(5′-GTTCT<u>GGA-</u>

TCC</u>AATGGCGAAGCCAATTGTC-3′) and *CAPOA*1-3(5′-TCGTA<u>GGATCC</u>ACTTCATCTTTTTCCGGACTTCATA-CC-3′), which added the *Bam*HI site to both ends of the amplified fragments. After digestion with *Bam*HI, the digested fragment was fused to the corresponding site of the N-terminus of the green fluorescence protein in pmsGFP vector [27]. Recombinant plasmid was then introduced to onion epidermal cells, using the Biolistic PDS-1000 gene delivery system (Bio-Rad, USA) (Fig. 7).

3. Results

3.1. Production of transgenic plants

In order to evaluate the effects of *CAPOA1* with regard to oxidative stress tolerance and disease resistance, the pepper *CAPOA1* cDNA, under control of the CaMV 35S promoter, was transformed into tobacco plants (Fig. 1A). Of eight independent primary transformants, four lines exhibited the expected 562 bp bands corresponding to the *CAPOA1* from the product of a genomic PCR, using gene-specific primers (Fig. 1B). Expression of *CAPOA1* in transgenic lines was also confirmed by Northern blot analysis, verifying that the four transgenic lines constitutively expressed high levels of *CAPOA1* transcription (Fig. 1C). T_1 seeds were collected via self-pollination, and used for further experiments.

3.2. Morphology and growth rates of the CAPOA1 transgenic plants

In order to ascertain whether or not the pepper ascorbate peroxidase-like 1 gene plays a crucial role in the growth and development of tobacco plants, we measured the root lengths of seedling plants and plant height/node numbers
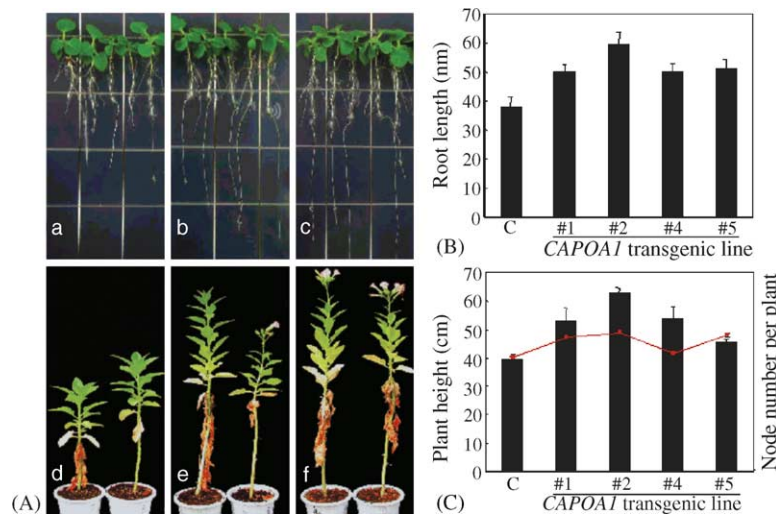

Fig. 2. Phenotype of the *CAPOA*1 transgenic tobacco plants. (A) (Upper panel) Root growth on MS medium supplemented with 50 mg/l kanamycin. a, control; b and c, *CAPOA*1 line #1 and #2, respectively. (Lower panel) Plant growth at the flowering stage: d, control; e and f, *CAPOA*1 line #1 and #2, respectively. (B) Root growth in MS + Kanamycin 50 mg/l medium. Data are expressed as the mean ± S.E. from three independent experiments. (C) Plant height and node number at the flowering stage. Data are expressed as the mean ± S.E. of 10–12 independent plants.
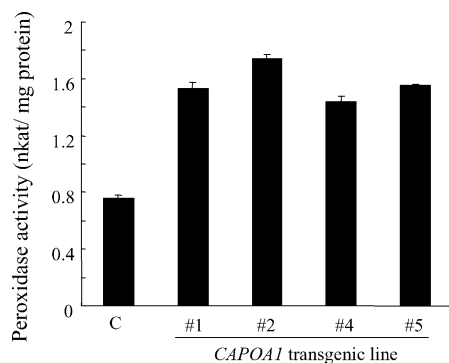
Fig. 3. Total peroxidase activity in the control and *CAPOA*1-transgenic tobacco plants. Data are expressed as the mean ± S.E. from three independent experiments.

during the flowering stage. All four of the transgenic lines proved to be 40% longer in root length, 35.53% taller in plant height, and had 15.42% more nodes than the control plants (Fig. 2). Interestingly, transgenic line #2, which exhibited the highest peroxidase activity level, exhibited more pronounced root length, as well as plant height and node number. These findings suggest that the overexpression of the pepper ascorbate peroxidase-like 1 gene may play an important role in the control of plant growth in seedlings, as well as in mature-stage plants.

3.3. Overexpression of CAPOA1 increases peroxidase activity

Total peroxidase activity was determined using fully expanded tobacco leaves of the control and all four transgenic T_1 lines. As expected, peroxidase activity in the *CAPOA*1-transgenic lines was twice as high as in the control (Fig. 3), indicating that the enhanced peroxidase activity in transgenic tobacco lines may be due to overexpression of the *CAPOA*1 gene.

3.4. Enhanced tolerance of transgenic plants to oxidative stress

When treated with MV, the *CAPOA*1-transgenic plants exhibited enhanced tolerance to oxidative stress in both the leaf disc and in the whole plant, relative to the control plants. After treatment with MV (50 or 100 μM), serious bleaching was observed in the control leaf discs, whereas bleaching was evident, but to a greatly reduced degree, in the transgenic plants. Thereafter, we determined total chlorophyll levels in both the MV-treated and non-treated leaf discs. After treatment with 50 or 100 μM MV, the transgenic lines evidenced 44.93 and 81.84% higher chlorophyll contents, respectively, compared to the controls. Interestingly, higher chlorophyll contents were also observed in transgenic plants compared to the control, even in non-treated leaf discs (Fig. 4A).

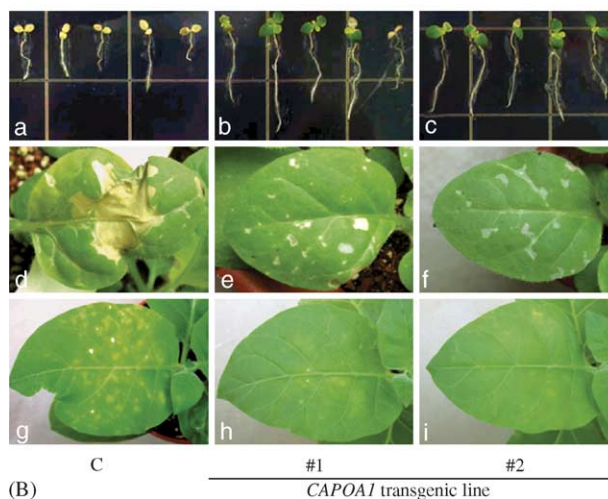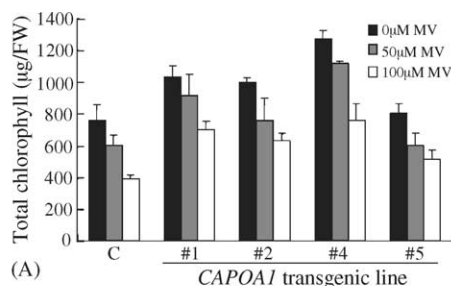Severe bleaching was observed in the control seedling plants within 4 days after treatment with 50 μM MV, and all

Fig. 4. Enhanced tolerance of the *CAPOA*1 transgenic tobacco plants to methyl viologen (MV). (A) Total chlorophyll content in leaf tissues. Black bar, without treatment; gray bar, treatment with 50 μM MV; white bar, treatment with 100 μM MV. Data are expressed as the mean ± S.E. from three independent experiments. (B) Oxidative stress tolerance of transgenic plants. (Upper panel) In vitro oxidative stress tolerance of transgenic plants. Ten-day-old seedlings were transferred to the MS medium supplemented with 50 μM MV: a, control; b and c, transgenic lines #1 and #2, respectively. (Middle panel) Effect of 50 μM MV on young (5-week-old) plants: d, control; e and f, transgenic lines #1 and #2, respectively. (Lower panel) Effect of 50 μM MV on mature (8- to 10-week-old) plants: g, control; h and i, transgenic lines #1 and #2, respectively.

the plants were dead before transfer to a recovery medium. The three transgenic lines, *CAPOA*1-1, *CAPOA*1-2, and *CAPOA*1-4, however, showed some slightly chlorotic symptoms, but remained alive, even under high oxidative stress conditions (Fig. 4B, upper panel). Finally, a total of 45–48% of the plants of the three transgenic lines survived after transfer to the recovery medium, whereas the transgenic line *CAPOA*1-5 showed chlorotic symptoms similar to those of the controls (data not shown). Furthermore, 5-week-old control plants were also found to be highly sensitive to the MV-meditated oxidative stresses, compared to the two transgenic lines: *CAPOA*1-1 and *CAPOA*1-2. In the control plants, typical necrotic spots appeared within 24 h of treatment, and several were observed to wilt, displaying numerous necrotic spots, 3 days after treatment. In contrast, transgenic plants also exhibited a few necrotic spots, but no wilt symptoms (Fig. 4B, middle panel). Moreover, in the mature plants, numerous chlorotic spots appeared in the controls, while
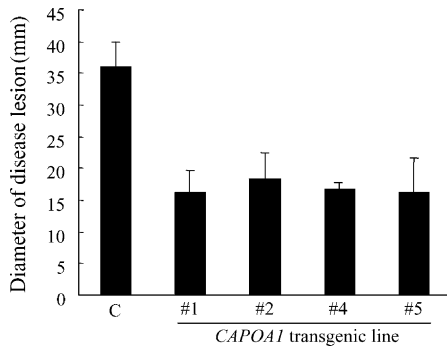
Fig. 5. Inhibition of disease development in the leaves of the *CAPOA*1 transgenic tobacco plants, infected by *Phytophthora nicotianae* isolate KACC40906. Disease development was recorded 5 days after infection on detached tobacco leaves. Data are expressed as the mean ± S.E. from three independent experiments.
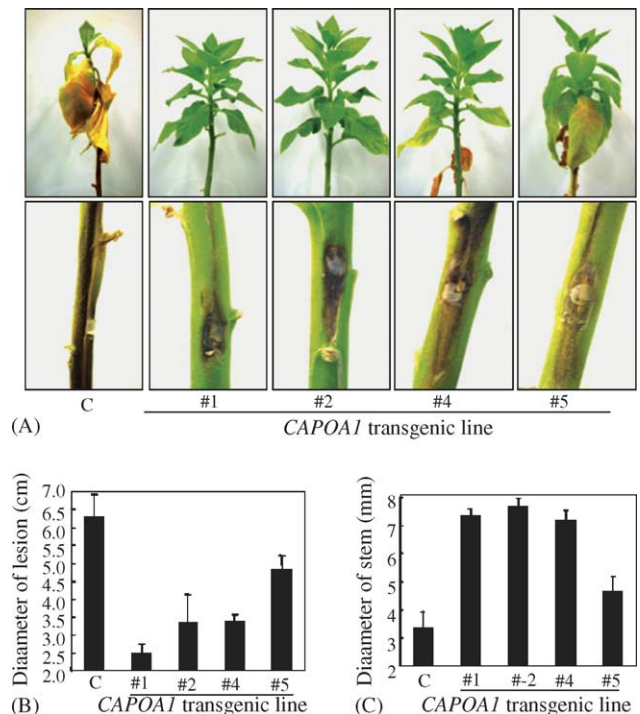

Fig. 6. Disease development in the *CAPOA*1 transgenic tobacco plants inoculated with *Phytophthora nicotianae* KACC40906 at the wounded stems of both control and transgenic lines. (A) Disease symptoms on the whole plant and inoculated stems. (B) Disease areas (cm in diameter) in the inoculated stems, 30 days after inoculation. (C) Measurement of stem diameters at the infected point after 30 days of infection. Data are expressed as the mean ± S.E. from three independent experiments.

transgenic plants also exhibited chlorotic spots, but to a greatly reduced degree (Fig. 4B, lower panel).

3.5. Enhanced resistance to pathogens

Studying order to determine whether or not the *CAPOA*1-overexpressing plants conferred resistance to the oomycete pathogen, *P. nicotianae*, we inoculated the center of a detached leaf surface with an oomycete plug. Five days after inoculation, disease symptoms appeared in all the tested leaves, indicating successful infections (data not shown). However, delayed disease development was observed in the transgenic lines, with a reduction rate of approximately 52% (Fig. 5).

More reliable and quantitative criteria for the detection of resistance to Black Shank disease in the *CAPOA*1 transgenic plants were verified in the stem infection assay. Visible small necrotic symptoms could be observed within 7 days after inoculation in all tested transgenic lines. However, the necrotic areas rapidly enlarged in the control plants. At 30 days after infection, the necrotic area measured 6.3 cm in diameter in the control plants, whereas transgenic plants exhibited greatly reduced necrotic zones (Fig. 6B). Hollow-shank symptoms also appeared at the stems of control plants, resulting in rapid chlorosis and wilting. However, such typical Black Shank symptoms were not observed in the three *CAPOA*1 lines #1, #2, and #4, whereas slight disease symptoms were detected in line #5 (Fig. 6A). The stem thickness at the infected point was also significantly reduced in the control plants (Fig. 6C). The two transgenic lines *CAPOA*1-1 and 1-2, which proved to be highly resistant to Black Shank disease, exhibited higher levels of both peroxidase activity (Fig. 3) and mRNA expression (Fig. 1C). Very similar results were also verified in young plant stems (5 to 6 weeks-old). These findings indicate that overexpression of the *CAPOA*1 gene in tobacco enhances resistance to the Black Shank pathogen *P. nicotianae*. However, the *CAPOA*1 transgenic plants did not appear to confer resistance to bacterial pathogens. We observed a slight inhibition of *R. solanacearum* growth, but no reduction of *P. syringae* growth, in the transgenic plants (data not shown).

3.6. Subcellular localization of CAPOA1 in onion epidermal cells

In order to identify the subcellular localization of *CAPOA*1, a reporter gene, coding for GFP, was fused to the C-terminus of the coding region of *CAPOA*1, and subjected to a transient assay using onion epidermal cells (Fig. 7). After biolistic bombardment, GFP fluorescence was detected outside of the cells, indicating that the CAPOA1 may be an apoplastic ascorbate peroxidase-like protein.

4. Discussion

It has been suggested [22] that the increased peroxidase activity manifest in both compatible and incompatible plant-pathogen interactions indicates that peroxidases are associated with non-specific defense responses, and provide enhanced resistance against both abiotic and biotic stresses. In this study, we examined the potential functions of the *CAPOA*1 gene in plants against oxidative stresses and pathogens, utilizing overexpressed tobacco lines, which had
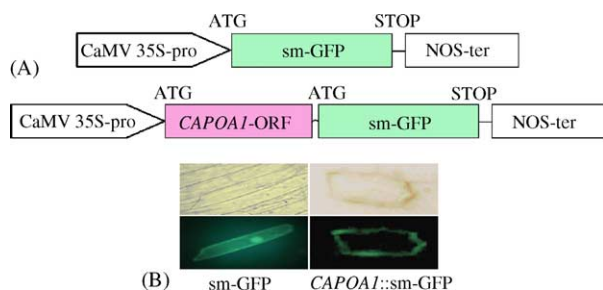
Fig. 7. Subcellular localization of CAPOA1 in onion epidermal cells. (A) Diagram of the vector constructs used. (Upper) only GFP as a control; (Lower) GFP fused to the C-terminus of the coding region of CAPOA1. (B) Subcellular localization of smGFP and CAPOA1::smGFP. (Upper) bright field image of onion epidermal cells; (Lower) UV-blue light excitation image of GFP and GFP-fused with CAPOA1 under fluorescence microscopy.

been previously isolated from pepper plants inoculated with the avirulent strain BV 5-4a of *X. campestris* pv. *vesicatoria* [10]. Although both *CAPOA*1 transcription and total peroxidase activity varied among all of the tested transgenic lines, total peroxidase activity in all the transgenic lines was twice as high as in the control plants. Previously, Kazan et al. [21] reported similar findings regarding the pathogenesis-related peroxidase gene (*Shpx* 6a) in tobacco and canola plants. Peroxidases have been demonstrated to play a role in several physiological processes, including growth control and cell expansion. As in the other previous reports, overexpression of *CAPOA*1 in the transgenic tobacco plants in this study showed no notable morphological abnormalities, but significantly higher plant heights were observed in transgenic lines. Our data are consistent with the findings of Pignocchi et al. [28], namely, that increased growth could be achieved in transgenic tobacco plants by overexpressing an apoplastic ascorbate oxidase. Expansion of tobacco protoplasts constitutively expressing ascorbate oxidase was also demonstrated by Kato and Esaka [29]. All these observations, taken together with our findings, constitute evidence that ascorbate oxidase/peroxidase plays a significant role in the growth and development of tobacco plants.

Moreover, *CAPOA*1-expressing transgenic plants showed enhanced tolerance to oxidative stress, which was induced by the application of MV, a superoxide (O_2^-) generating compound. When leaf discs were exposed to MV (50 and 100 μM), significant degradation of chlorophyll was observed in the control plants, and to a lesser degree, or not at all, in the transgenic lines. This phenomenon may be attributable to chlorophyll's pronounced susceptibility to ROS-mediated damage. In the electron transfer system, electrons released from the photosynthetic machinery can easily react with relatively high concentrations of O_2 in the chloroplast [2]. Plants are, then, required to activate antioxidant defense mechanisms in order to protect them from the consequent oxidative stresses. Overexpression of ascorbate peroxidase may be required to trigger such defense mechanisms via the conversion of hydrogen

peroxide to water. In response to several stress conditions, much higher increased activity of APX was observed compared with other antioxidants, such as catalase, indicating that it has a role in detoxification of oxygen-activated species [6]. It is also reported that the increased expression of APX activity was observed in the ozone-exposed *Arabidopsis*, while other antioxidant enzymes, such as monodehydroascorbate reductase, dehydroascorbate reductase and glutathione reductase, were unaffected [30].

In this study, we demonstrated that the overexpression of *CAPOA*1 in transgenic tobacco confers enhanced tolerance to MV-mediated oxidative damage, both in seedlings and in mature plants, as well as in leaf discs. Badawi et al., [31] recently reported that the overexpression of cytosolic ascorbate peroxidase in tobacco chloroplasts increased APX activity 3.8-fold, reduced the toxicity of H_2O_2, and enhanced tolerance to active oxygen species generating paraquat and sodium sulphite. Overexpression of other several antioxidant genes, most notably, Fe- and/or Cu/Zn-superoxide dismutase, glutathione peroxidase in tobacco chloroplasts also enhanced oxidative stress tolerance [32–34]. Though there are several reports on transgenic plant overexpressing antioxidant genes enhanced tolerance to oxidative stresses, however, Torsethaugen et al. [35] reported transgenic tobacco with enhanced APX activity had no tolerance against ozone damage.

Previous reports have attempted to delineate the enhanced resistance effects of the pathogenesis-related peroxidases in transgenic tobacco plants to the oomycete pathogen, *P. nicotianae* [21,22]. However, these reports also noted that these peroxidases conferred no resistance against the fungal pathogens *Erysiphe cichoracearum* in tobacco, and *Fusarium oxysporum* in tomato [36,37]. This suggests that, although some peroxidases are clearly involved in host resistance to certain fungi, not all the peroxidases play such a defensive role. In our findings, we also verified that *CAPOA*1-transformed tobacco plants exhibited enhanced resistance to *P. nicotianae*. Interestingly, the *CAPOA*1-transgenic lines with high levels of mRNA expression and high peroxidase activity exhibited increased resistance to the oomycete pathogen. However, slight, but not significant, resistance against the bacterial pathogen *R. solanacearum* was observed, and no resistance to *P. syringae* pv. *tabaci* was observed (data not shown). Although the precise defensive role of ascorbate peroxidase has not yet been adequately worked out, it is suggested that overexpression of the *CAPOA*1 gene is very effective in reducing pathogen growth at the infection sites of transgenic plants. To further clarify the functions of this gene, we performed subcellular localization of *CAPOA*1. Subcellular localization revealed that the signal of GFP fused to *CAPOA*1 could be detected in the extracellular region of the cell, suggesting its possible role in the inhibition of pathogenic growth. The expression of *PR*-1a and *thaumatin* genes increased in the *CAPOA*1-overexpressing lines which were observed by RT-PCR analysis, whereas the expressions of *PR*-2, *PR*-*Q*, and *PR*-4

were unaffected (data not shown). *PR*-1, which is most commonly used as a marker for systemic acquired resistance, is a dominant group of *PR* proteins. Alexander et al. [38] reported that the constitutive expression of *PR*-1*a* in transgenic tobacco enhanced tolerance to the oomycete pathogens *P. tabacina* and *P. parasitica* var. *nicotianae*. Overexpression of the thaumatin-like protein gene was demonstrated to enhance tolerance to *Rhizoctonia solani*, which causes sheath blight disease in rice [39]. Therefore, disease resistance in this study may be due to the increased expressions of *PR*-1 and *thaumatin* genes induced in the transgenic plants. In order to produce commercial lines for oxidative stress-tolerant and disease-resistant plants, such a molecular breeding technique will be necessary for the development of genetically engineered, agriculturally important crops, such as tomato and potato, because *CAPOA*1 is a novel gene for developing transgenic plants with enhanced tolerance to oxidative stresses, and resistance to oomycete pathogens.

Acknowledgements

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