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Functional Roles of the Pepper Ascorbate Peroxidase1 (*CaPOA1*) in the Abiotic Stress Tolerance in Plants*

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식물의 비생물적 스트레스 내성발현에서 고추 아스코르베이트 퍼옥시다아제1(*CaPOA1*)의 기능적 역할*

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ABSTRACT

Plants are equipped with various enzymatic antioxidant machinery to relieve oxidative stress induced by various abiotic stresses. Among the various antioxidant enzymes, ascorbate peroxidase plays an important role in the detoxification of reactive oxygen species (ROS) produced during various oxidative stresses. In a previous study, *Capsicum annuum ascorbate peroxidase 1 (CaPOA1)* was identified to be highly expressed in pepper leaves treated with hydrogen peroxide (H_2O_2) and salicylic acid and inoculated with different pathogens. In this study, we investigated *in planta* function of *CaPOA1* during different oxidative stress conditions induced

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by high salinity, osmotic stress and methyl viologen (MV) treatment using the virus-induced gene silencing (VIGS) technique in pepper and ectopic overexpression in Arabidopsis plants. Expression of *CaPOA1* in pepper plants was induced by high salinity (200 and 400 mM NaCl), osmotic stress (200 and 400 mM mannitol) and MV-induced oxidative stress (0.5 and 1 μM MV). VIGS of *CaPOA1* in pepper plants, drastically reduced the expression of *CaPOA1* and reduced the enhanced chlorosis and lipid peroxidation under these stress conditions, suggesting that *CaPOA1* expression is involved in tolerance to different oxidative stresses. In contrast, *CaPOA1* overexpression (OX) in transgenic *Arabidopsis thaliana* (*CaPOA1*-OX) plants conferred enhanced tolerance (resistance) to these stress conditions. Notably, *CaPOA1* overexpression significantly enhanced total peroxidase activity in transgenic Arabidopsis (*CaPOA1*-OX) plants, but reduced the transgenic Arabidopsis root length under normal growth conditions. Collectively, these findings provide evidence for the involvement of *CaPOA1* in the tolerance (resistance) of pepper plants to various abiotic stresses as well as root growth and development of plants.

Key words: High salinity, Osmotic stress, Oxidative stress, Ascorbate peroxidase gene, *Capsicum annuum*, Transgenic *Arabidopsis thaliana*, Virus-induced gene silencing,

초 록

식물은 다양한 비생물적 스트레스에 의해 유발되는 산화 스트레스를 완화하기 위해 다양한 효소적 항산화 기구(machinery)를 갖추고 있다. 다양한 항산화 효소 중에서 아스코르브산 과산화효소는 다양한 산화 스트레스 동안 생성되는 활성산소(ROS)의 해독에 중요한 역할을 한다. 이전 연구에서 고추 아스코르브산 과산화효소 유전자(*Capsicum annuum ascorbate peroxidase 1, CaPOA1*)는 과산화수소(H_2O_2)와 살리실산(salicylic acid)을 처리하거나 다양한 병원체를 접종한 고추 잎에서 높게 발현되는 것으로 확인되었다. 이 연구에서는 고추의 바이러스 유도 유전자 침묵(VIGS)기술과 애기장대 (Arabidopsis) 식물에서 이소성 과발현(ectopic overexpression) 형질전환기술을 사용하여 높은 염도(salinity), 삼투압(osmotic) 스트레스 및 메틸 바이올로겐(MV, methyl viologen) 처리에 의해 유도된 상이한 산화 스트레스(oxidative stress) 조건에서 *CaPOA1*의 식물에서의 기능을 조사했다. 고추

식물에서 *CaPOA1* 유전자의 발현은 고염도(200 및 400mM NaCl), 삼투압 스트레스(200 및 400mM mannitol) 및 MV 유발 산화스트레스(0.5 및 1μM MV)에 의해 유도되었다. *CaPOA1* 유전자를 타겟으로 VIGS를 유도한 고추 식물에서는 이러한 스트레스 조건에서 *CaPOA1*의 발현이 급격히 감소되고 황화(chlorosis)와 지질 과산화(lipid peroxidation)를 증가시키는 것으로 나타나 *CaPOA1* 발현이 다양한 산화스트레스에 대한 내성에 관여함을 시사한다. 이에 반하여, 형질전환 *Arabidopsis thaliana* (*CaPOA1*-OX) 식물은 *CaPOA1*이 과발현(OX, overexpression)되어 이러한 스트레스 조건에 대해 내성(저항성)의 증가를 보였다. 특히, *CaPOA1* 과발현은 형질전환 애기장대(*CaPOA1*-OX) 식물에서 총 과산화효소(peroxidase) 활성을 크게 증가시키지만 정상적인 성장 조건에서 이 형질전환 애기장대 뿌리 길이를 감소시켰다. 종합적으로, 이러한 연구결과는 식물의 뿌리 성장 및 발달뿐만 아니라 다양한 비생물적 스트레스에 대한 고추 식물의 내성(저항성)에 *CaPOA1*이 관여한다는 증거를 제시해 주고 있다.

주제어: 고염도, 삼투압스트레스, 산화스트레스, 아스코르브산 과산화효소 유전자, 고추, 형질전환 애기장대, 바이러스 유도 유전자침묵

목 차

I. Introduction

As sessile organisms, plants respond and survive persistently and sophisticatedly to a variety of environmental and biotic stressors. Reactive oxygen species (ROS) are produced by plants and play an important role as signaling molecules to regulate plant growth, development and responses to biotic and abiotic stresses (Tsukagoshi, 2016; Waszczak et al., 2018; Huang et al., 2019; Anderson and Kim, 2021; Considine

and Foyer, 2021). In plants, ROS, such as singlet oxygen ($^1O_2$), superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (HO.), are produced as signaling molecules as well as by-products of aerobic metabolism (Waszczak et al., 2018). They respond to various environmental stresses, such as UV exposure, high and low temperature, salinity, drought and pathogen attack (Das and Roychoudhury, 2014). The balance between ROS generation and detoxification is important for the progression of several basic biological processes, including cell proliferation and differentiation (Tsukagoshi et al., 2010; Zafra et al., 2010; Tripathy and Oelmuller, 2012). However, higher production of ROS induced by an imbalance between ROS production and antioxidant capacity can lead to oxidative stress, which leads to destruction of cell organelles, membrane lipid peroxidation, degradation of biological macromolecules and ultimately programmed cell death.

Both enzymatic and non-enzymatic antioxidant defense machinery protect plants from oxidative damages. Among them, the enzymatic antioxidant defense machinery include superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), glutathione peroxidase (GPX), glutathione reductase (GR), glutathione S-transferases (GST), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), and dehydroascorbate reductase (DHAR), which work together to efficiently scavenge ROS (Gill and Tuteja, 2010; Rajput et al., 2021; Nehela et al., 2021). In particular, APX is a member of heme-containing peroxidases family and catalyze the conversion of H_2O_2 to H_2O, using ascorbate as a specific electron donor (Caverzan et al., 2012). In other studies, expression of the *APX* gene is stimulated by various oxidative stress-inducing conditions, such as high salinity, osmotic stress, high and low temperature, etc. Altered expression of *APX* by overexpression or mutation significantly enhances or inhibits plant tolerance to oxidative stress, respectively (Teixeira et al., 2006; Maruta et al., 2010; Bonifacio et al., 2011; Zhang et al., 2022). These results suggest that

APX plays a crucial role for ameliorating oxidative stress induced by adverse environmental conditions.

In our previous study, we have isolated and identified pepper ascorbate peroxidase 1 (*CAPOA1*) from pepper leaves inoculated with *Xanthomonas campestris* pv. *vesicatoria (Xcv)* (Do et al., 2003). *CaPOA1* expression were observed at low levels in the roots, and green and red fruits of pepper without elicitor treatment. However, in the incompatible interaction of pepper plants with avirulent *Xcv, CaPOA1* expression was strongly induced up to 12 h after inoculation (hpi), but its expression distinctly decreased at 18-30 hpi. By contrast, significantly enhanced accumulation of H_2O_2 was observed at 18-30 hpi in pepper leaves inoculated with avirulent *Xcv*. This suggests that inhibition of *CaPOA1* expression is required to enhance the accumulation of H_2O_2, which is important for further hypersensitive reactions. Other oxidative stress-inducing conditions, such as H_2O_2 and salicylic acid treatment, and *Phytophthora capsici* and *Colletotrichum gloeosporioides* infection, also markedly induced *CaPOA1* expression in pepper plants (Do et al., 2003).

To understand biological function of *CaPOA1* during different oxidative stress conditions induced by high salinity, osmotic stress and methyl viologen (MV, the herbicide Paraquat) treatment, loss-of-function and gain-of-function assays were performed using the virus-induced gene silencing (VIGS) in pepper and transgenic overexpression in Arabidopsis plants, respectively. VIGS of *CaPOA1* significantly reduced the tolerance of pepper leaves to oxidative damage induced by high salinity, osmotic stress and MV-induced oxidative stress; however, *CaPOA1* overexpression significantly enhanced the tolerance of transgenic Arabidopsis plants. Taken together, these results suggest that *CaPOA1* actually plays an important role in scavenging ROS generated during different oxidative stresses to enhance plant tolerance.

II. Materials and Methods

1. Plant materials and growth conditions

Pepper (*Capsicum annuum* L., cv. Nockwang) plants were grown in plastic trays (55×35×15cm) containing steam-sterilized soil mixure (peat moss, perlite, and vermiculite; 5:3:2, v/v/v) and loam (1:1, v/v) at 28℃, 16 h a day length at a light intensity of 70 μmol photons $m^{-2}s^{-1}$. Pepper plants at the six-leaf stage were used for pathogen infection as well as abiotic elicitor and environmental stress treatment.

Arabidopsis thaliana ecotype Columbia (Col-0) plants were grown at 24℃ with a photosynthetic flux of 130 μmol photons m^{-2} s^{-1} for 16 h (a long day condition) or 12 h (a short day condition) light and 60% relative humidity in a controlled environmental chamber. Plants were grown in pots containing vermiculite, peat moss and perlite (1:1:0.5, v/v/v). Prior to sowing, seeds of wild-type (Col-0) and *CaPOA1*-OX plants were sterilized with a 2% sodium hypochlorite solution and then submerged at 4℃ for 3 days to overcome dormancy.

2. RNA isolation and RNA gel blot analysis

Total RNA was extracted from pepper and Arabidopsis leaves using TRIzol Reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. To generate a gene-specific probe, the coding region of the *CaPOA1* (*Capsicum annuum Ascorbate Peroxidase1*) gene was amplified using the *CaPOA1* primers (forward, 5'-CTTTGATGGTCCATGGACAAAGG-3') and (reverse, 5'-CTTCATCTTTTTCCGGACTT-3'). The amplified PCR product was labeled with ^{32}P using a random priming kit (Boehringer Mannheim, Mannheim, Germany). Agarose gel electrophoresis, RNA transfer, and hybridization with *CaPOA1* probes were performed according to the standard procedures.

3. RT-PCR analysis

The expression levels of *CaPOA1* were examined by RT-PCR during the abiotic stress treatment. The empty vector control (TRV:00) and *CaPOA1*-silenced (TRV:00) leaf discs were treated with the indicated concentrations of sodium chloride (NaCl), mannitol and methyl viologen (MV) for 12 h. Total RNA was extracted as previously described and 2 μg of total RNA were used for cDNA synthesis, as previously described (Choi et al., 2007). An aliquot (1 μl) of the reverse transcription (RT) reaction product was used for RT-PCR analysis of the expression of *CaPOA1* using the gene-specific primers described above.

4. Virus-induced gene silencing (VIGS)

The TRV-based VIGS system was used for *CaPOA1* silencing in pepper plants as previously described (Liu et al., 2002). The C-terminal region of *CaPOA1* open reading frame (349 bp) was amplified by PCR using primers (forward, 5'-CTTTGATGGTCCATGGACAAAGG-3'; reverse, 5'-CTTCATCTTTTTCCGGACTT-3') and cloned into the vector pTRV2 to yield pTRV2:*CaPOA1*. *Agrobacterium tumefaciens* strain GV3101 (OD_{600}=0.2 for each) carrying either pTRV1 or pTRV2:*CaPOA1* was co-infiltrated into fully expanded cotyledons of pepper plants. *Agro*-infiltrated pepper plants were placed in a growth room at 25℃ with a 16h-light/8h-dark photoperiod for growth and virus spread.

5. Arabidopsis Transformation

Transgenic Arabidopsis plants overexpressing the *CaPOA1* gene were generated using the floral dipping method (Clough and Bent, 1998). Three putative transgenic Arabidopsis lines (T1) containing *35S:CaPOA1* were selected by planting seeds in Murashige and Skoog

medium (Duchefa) containing 50 mg L^{-1} kanamycin.

6. Treatment with abiotic elicitors and environmental stresses

Leaf discs excised from the gene-silenced pepper leaves using a cork borer were floated in NaCl or mannitol solutions at concentrations of 0, 100, 200, 300, 400 and 500 mM and maintained for 3 days in continuous white light of 70 μmol photons $m^{-2}s^{-1}$ at 26℃ to test salinity and dehydration stress, respectively. The total chlorophyll content was measured with a spectrophotometer after extraction with 80% (v/v) acetone. For oxidative stress experiments, leaf discs were floated in MV solutions at concentrations of 0, 0.25, 0.5, 0.75, 1.0 and 1.25 μM for 3 days in continuous white light of 70 μmol photons m^{-2} s^{-1} at 26℃.

To examine the germination rate of *CaPOA1* transgenic plants under various abiotic stress conditions, sterile wild-type (WT) and *CaPOA1*-OX seeds were vernalized for 3 days at 4℃ to synchronize germination and then placed on 1× Murashige and Skoog (MS) medium containing 500 mM NaCl, 500 mM mannitol, or 1 μM MV; Germination (emergence of radicals) was scored 7 days after treatment.

The 7-day-old wild-type and *CaPOA1*-OX seedling plants were floated in NaCl, mannitol and MV solutions at the indicated concentrations and maintained for 14 days to test the tolerance of transgenic plants to high salinity, osmotic and oxidative stresses, respectively. The seedling plants was weighed and used to determine the total chlorophyll content.

7. Determination of total chlorophyll content

Chlorophyll content was measured spectrophotometrically according to the formula ($Ca+b = 5.24 \times A_{664} + 22.24 \times A_{648}$), where C is the chlorophyll concentration (Ca+b) in micrograms per mL and A is the

absorbance (An et al., 2008). Each measurement was performed using five seedling plants and the experiments were repeated three times with similar results.

8. Lipid peroxidation

The progression of lipid peroxidation was assessed by measuring thiobarbituric acid reactive substances (TBARS), as previously described (Heath and Packer, 1968; Choi and Hwang, 2012). Six leaf discs from the gene-silencing pepper leaves and three Arabidopsis seedling plants were homogenized in a solution of 0.5 % (w/v) thiobarbituric acid (TBA) in 20 % (w/v) trichloroacetic acid (TCA). The mixture was boiled at 95℃ for 30 min and cooled on ice for 5 min. After centrifugation at 13,000 *g* for 10 min, the absorbance of the supernatant was measured at 600 nm and was subtracted from the absobance at 532 nm, and the malondialdehyde (MDA) concentration was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}.

9. Peroxidase activity assay

Total peroxidase activities of wild-type (WT) and *CaPOA1*-OX Arabidopsis plants were assessed as previously described (Hammerschmidt et al. 1982; Choi et al., 2007). Briefly, total proteins were extracted by homogenizing Arabidopsis leaves with a mortar and pestle supplemented with 5 volumes of extraction buffer (0.1 M sodium phosphate buffer, pH 6.0, 0.5 M sucrose). The homogenates were centrifuged at 10,000 *g* and 4℃ for 20 min. The clear supernatant was used for peroxidase activity assay according to the method of Hammerschmidt et al. (1982). Peroxidase activity was determined using guaiacol as the hydrogen donor. Leaf extract (100 μL) was added to the reaction mixture (0.1 M sodium phosphate buffer, pH 6.0, 0.25% guaiacol, 1 M

H_2O_2), and then incubated at 25℃ for 5 min. Peroxidase activity was determined by an increase in A_{470} min^{-1} mg^{-1} protein. Peroxidase activity of leaf extracts was calculated using the molar extinction coefficient of tetraguaiacol (2.66 x 10^4 mol^{-1} cm^{-1}) and the enzyme activity was expressed as nanokats per milligram of total proteins.

III. Results

1. Reduced tolerance of *CaPOA1*-silenced pepper to salinity and osmotic stresses

In a previous study, we demonstrated that the pepper ascorbate peroxidase gene *CaPOA1* (accession number AF442387) was differentially expressed in pepper against bacterial spot and anthracnose pathogen infection and hydrogen peroxide (H_2O_2) treatment (Do et al., 2003). Further transformation studies of gene overexpression in tomato also showed that constitutive expression of two pathogenesis-related genes enhances resistance of tomato plants to the oomycete pathogen *Phytophthora capsici* (Sarowar et al., 2006). To analyze the loss-of-function of *CaPOA1* during abiotic stress tolerance in pepper plants, we used the tobacco rattle virus (TRV)-based VIGS technique (Liu et al., 2002; Chung et al., 2004). To investigate the role of *CaPOA1* expression during high salinity stress, leaf discs from the gene-silenced plants were exposed to various concentrations of sodium chloride (NaCl) (Figure 1). As shown in Figure 1A, empty vector control leaves (TRV:00) strongly accumulated *CaPOA1* transcripts after high salt stress treatment; however *CaPOA1* transcripts were detected at a slightly visible level in the gene-silenced leaves 12 h after 200 and 400 mM NaCl treatment,

suggesting that the silencing was effective for *CaPOA1* gene in pepper plants. Interestingly, leaf discs from *CaPOA1*- silenced plants showed more severe bleaching phenotype compared to the control plants during 7-day incubations in increasing concentrations of NaCl (Figure 1B). The chlorophyll content in the leaf discs of gene-silenced plants was measured after 7 days to quantify the phenotypic differences. The loss of chlorophyll pigment was significantly higher in the *CaPOA1*-silenced plants compared to the empty vector control plants after treatment with various concentrations of NaCl (Figure 1C). To determine cellular damage induced by high salinity stress, the level of lipid peroxidation was also investigated by measuring the accumulation of thiobarbituric acid reactive substances (TBARS) in the leaf discs incubated with increasing concentrations of NaCl (Figure 1D). Interestingly, leaf discs from CaPOA1-silenced pepper showed significantly enhanced levels of lipid peroxidation without NaCl treatment (Figure 1D; 0 mM). In addition, *CaPOA1*-silenced plants showed significantly higher levels of lipid peroxidation, compared to empty vector control plants after the treatment of various concentrations of NaCl (Figure 1D). This suggests that CaPOA1 silencing significantly reduced the tolerance of pepper plants to high salinity stress.

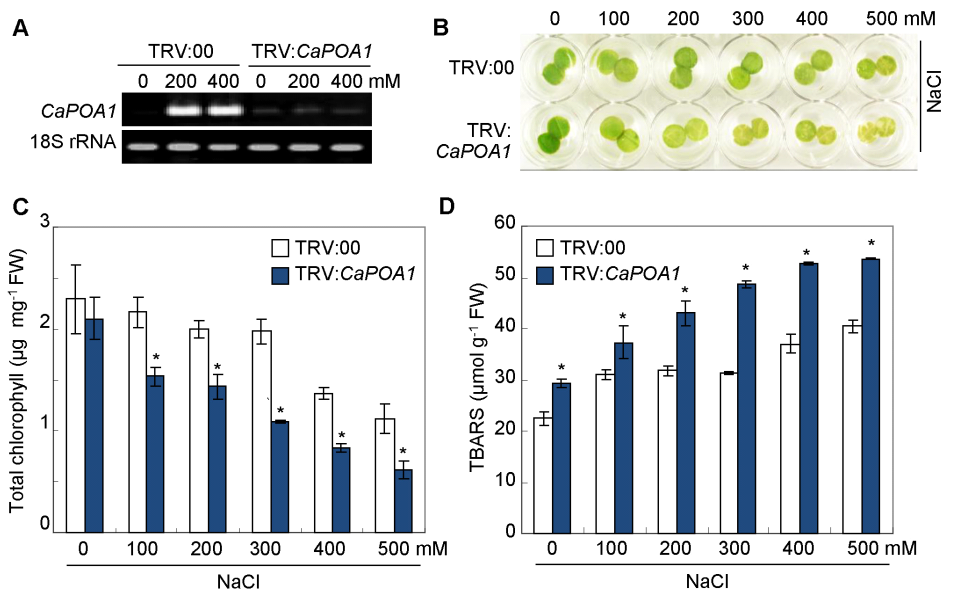

Figure 1. Decreased tolerance of *CaPOA1*-silenced pepper plants to salinity stress. (A) RT-PCR analyses of the expression of *CaPOA1* in empty vector control (TRV:00) and CaPOA1-silenced (TRV:*CaPOA1*) pepper leaves 12 h after NaCl treatment. Levels of pepper 18S rRNA were visualized as internal controls. (B) Chlorosis phenotype, (C) chlorophyll content and (D) lipid peroxidation level of leaf discs of the gene-silenced pepper plants in response to high salinity stress. Leaf discs from the gene-silenced plants were floated in NaCl solutions of different concentrations for 72 h under continuous light (70 μmol m^{-2} s^{-1}) at 26℃. Asterisks indicate significant differences compared to empty vector control leaves (Student's t test, $P < 0.05$).

Altered tolerance of *CaPOA1*-silencing plants to osmotic stresses was also examined in the increasing concentrations of mannitol (Figure 2). Treatment of leaf discs of empty vector control plants (TRV:00) with 200 and 400 mM mannitol induced *CaPOA1* expression (Figure 2A). However, *CaPOA1*-silenced plants markedly compromised the accumulation of the corresponding transcripts upon mannitol treatment. Treatment of leaf discs from *CaPOA1*-silenced plants with varying concentrations of mannitol resulted in a more sever bleaching phenotype after incubation for 7 days compared to the empty vector control plants (Figure 2B). Measurement of chlorophyll content in the leaf discs of gene-silencing plants showed the significantly enhanced loss of chlorophyll pigment in *CaPOA1*-silenced leaves compared to the empty vector

control (Figure 2C). Lipid peroxidation in *CaPOA1*-silenced leaves was also significantly higher than that in control leaves, suggesting that a higher level of cellular damage occurred in the gene-silenced leaves after mannitol treatment (Figure 2D). These results indicate that expression of the *CaPOA1* gene play an important role in high salinity and osmotic stress tolerance of pepper plants.

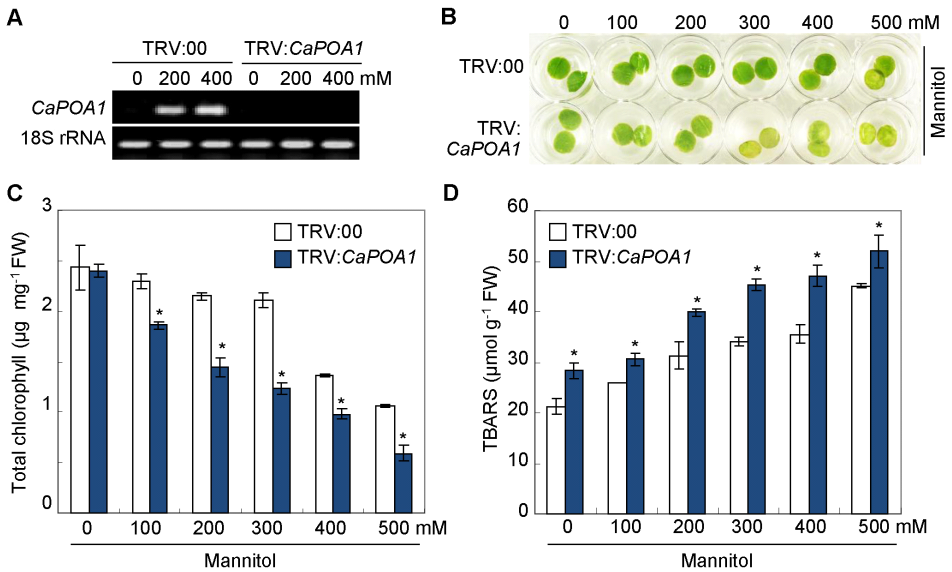

Figure 2. Decreased tolerance of *CaPOA1*-silenced pepper plants to osmotic stress. (A) RT-PCR analyses of the expression of *CaPOA1* in empty vector control (TRV:00) and *CaPOA1*-silenced (TRV:*CaPOA1*) pepper leaves 12 h after mannitol treatment. Levels of pepper 18S rRNA were visualized as internal controls. (B) Chlorosis phenotype, (C) chlorophyll content and (D) lipid peroxidation levels of leaf discs of the gene-silenced pepper plants in response to high osmotic stress. Leaf discs of gene-silenced plants were floated in mannitol solutions of different concentrations for 72 h under continuous light (70 μmol m^{-2} s^{-1}) at 26℃. Asterisks indicate significant differences compared to empty vector control leaves (Student's t test, $P < 0.05$).

2. Reduced tolerance of *CaPOA1*-silenced pepper to methyl viologen (MV)

To address the role of *CaPOA1* in oxidative stress, leaf discs of empty vector control and *CaPOA1*-silenced plants were treated with the O_2^--generating herbicide methyl viologen (MV). To evaluate the effectiveness of VIGS, *CaPOA1* transcript levels were examined by reverse transcription (RT)-PCR in empty vector control (TRV:00) and *CaPOA1*-silenced (TRV:*CaPOA1*) pepper leaves 12 h after MV treatment (Figure 3A). *CaPOA1* transcripts were strongly induced in empty vector control leaves upon treatment with 0.5 and 1.0 μM MV. However, *CaPOA1* silencing failed to induce *CaPOA1* expression. More importantly, silencing of the *CaPOA1* gene led to decreased resistance to MV-induced oxidative stress with accelerated loss of chlorophyll pigment (Figure 3B). *CaPOA1*-silenced pepper leaves (TRV:*CaPOA1*) began to exhibit a distinct bleaching phenotype after treatment with 0.5 μM MV, which levels were comparable to empty vector control leaves (TRV:00) treated with 1.0 μM MV. Measuring the chlorophyll content of MV-treated leaf discs from the gene-silencing plants revealed a significant decrease in tolerance of *CaPOA1*-silenced leaves (Figure 3C). Treatment of leaf discs with 0.25 μM MV did not induce significant difference in chlorophyll levels between control and silenced leaves, whereas higher concentrations of MV treatment ($\rangle$ 0.5 μM) significantly decreased chlorophyll levels in *CaPOA1*-silenced leaves compared to empty vector controls. Measurements of lipid peroxidation levels in the leaf discs incubated with increasing concentrations of MV also revealed increased cellular damage in *CaPOA1*-silenced leaves by MV treatment (Figure 3D).

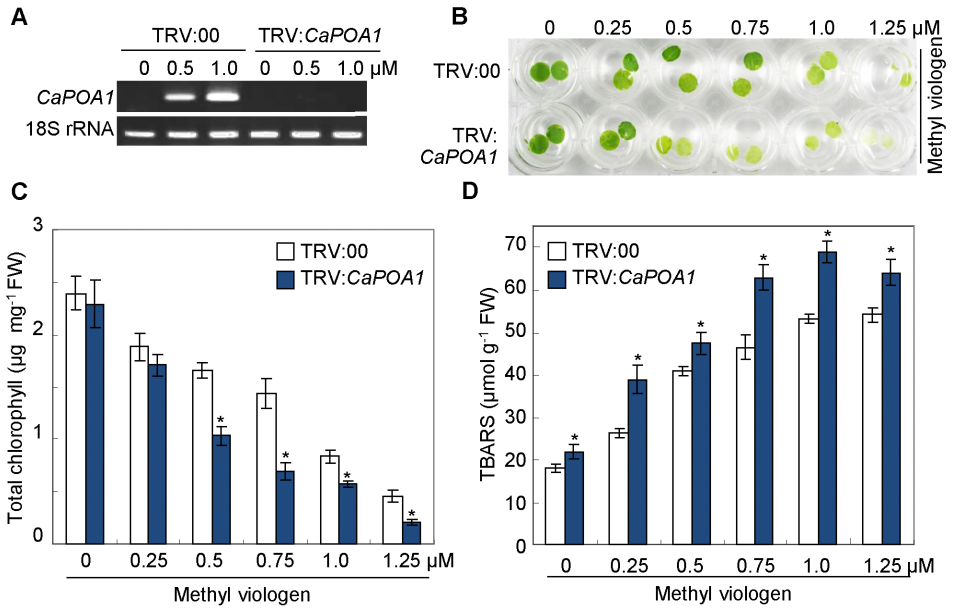

Figure 3. Decreased tolerance of *CaPOA1*-silenced pepper plants to methyl viologen (MV)-induced oxidative stress. (A) RT-PCR analyses of the expression of *CaPOA1* in empty vector control (TRV:00) and *CaPOA1*-silenced (TRV:*CaPOA1*) pepper leaves 12 h after MV treatment. Levels of pepper 18S rRNA was visualized as internal controls. (B) Chlorosis phenotypes, (C) chlorophyll contents and (D) lipid peroxidation levels of leaf discs of the gene-silenced pepper plants in response to MV. Leaf discs of gene-silenced plants were floated in MV solutions of different concentrations at 26℃ under continuous light (70 μmol m^{-2} s^{-1}) for 72 h. Asterisks indicate significant differences compared to empty vector control leaves (Student's t test, $P < 0.05$).

3. Altered root growth and development in *CaPOA1*-overexpression (OX) Arabidopsis plants

To determine the *in planta* functions of *CaPOA1* during abiotic stress tolerance, we generated transgenic *Arabidopsis thaliana* plants overexpressing the *CaPOA1* gene. To generate transgenic Arabidopsis plants, the open reading frame (ORF) of the *CaPOA1* gene was integrated between the *CaMV* 35S promoter and the *nos* terminator region of the pBIN35S binary vector. Arabidopsis ecotype Columbia (Col-0) plants were transformed using the 35S:*CaPOA1* construct according to the floral dipping method. Constitutive expression of *CaPOA1* in three independent *CaPOA1*-overexpression (OX) lines was

confirmed by RNA gel blot analyses, but not in wild-type (WT) plants (Figure 4A). Total peroxidase activity was assessed in wild-type and *CaPOA1*-OX plants, as previously described by Hammerschmidt et al. (1982) (Figure 4B). Wild-type plants contained low levels of total peroxidase activity, but all *CaPOA1*-OX plants tested exhibited approximately 10-20 fold higher total peroxidase activity compared to WT plants. As expected, the enhanced total peroxidase activity of *CaPOA1*-OX plants was proportional to the amount of *CaPOA1* transcription levels as observed by RT-PCR (Figure 4A and 4B).

Phenotypic differences in root growth between WT plants and *CaPOA1*-OX lines were observed during the growth on the Murashige and Skoog (MS) media. The *CaPOA1*-OX seedling plants showed the increased number and growth of lateral roots, but reduced growth of primary roots (Figure 4C). As shown in Figure 4D, overexpression of *CaPOA1* in Arabidopsis plants significantly reduced the growth of primary roots. More importantly, the extent of root growth changes was proportional to the level of *CaPOA1* expression and total peroxidase activity, resulting in longer lateral roots and shorter primary roots. This suggests that overexpression of *CaPOA1* in Arabidopsis plants negatively affects normal plant root growth and development.

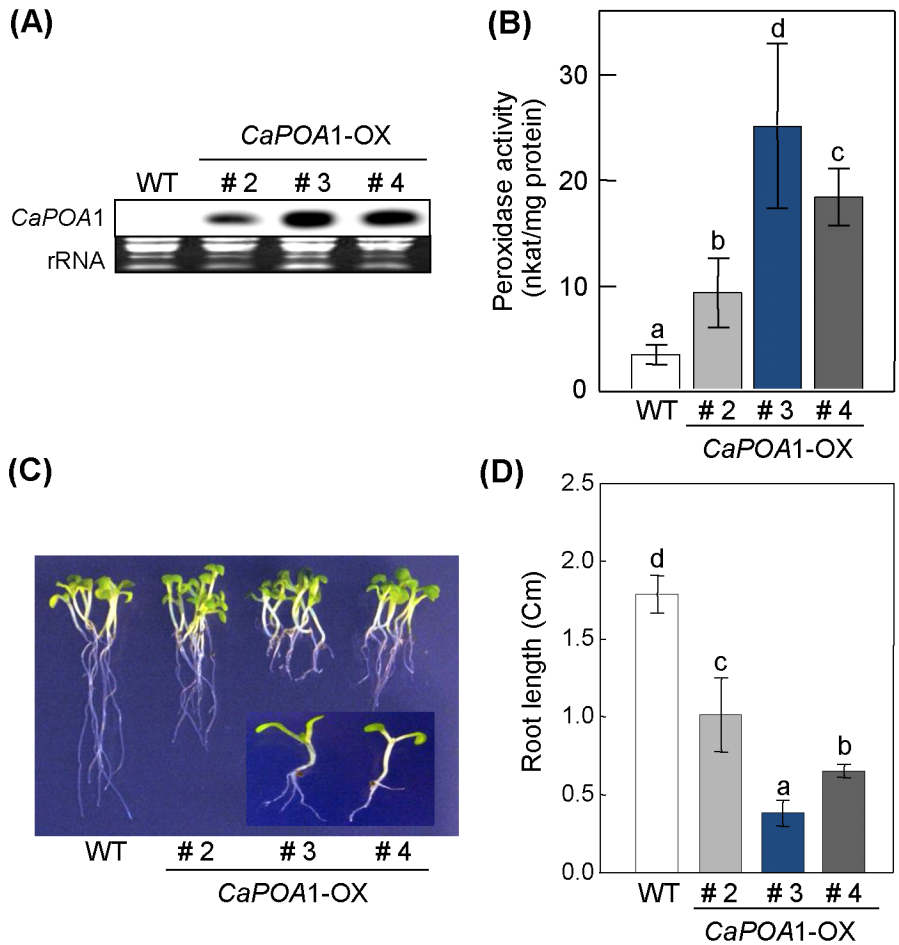

Figure 4. Generation of Arabidopsis *CaPOA1*-overexpression (OX) transgenic Arabidopsis plants. (A) RNA gel blot analyses of the expression of the *CaPOA1* in leaves of wild-type (WT) and *CaPOA1*-OX Arabidopsis (#2, #3 and #4) plants. Equal loading of total RNA (10 μg per lane) was verified by visualizing rRNA on ethidium bromide-stained gels. (B) Spectrophotometric quantification of total peroxidase activity in Arabidopsis WT and *CaPOA1*-OX plants. Data are the means ± standard deviations from five independent experiments. (C) Growth comparison of WT and *CaPOA1*-OX transgenic plants grown on MS plates for 7 days. (D) Reduced primary root length of *CaPOA1*-OX transgenic Arabidopsis plants grown on MS plates. Different letters indicate significant differences, as determined by the least significant difference test (P = 0.05).

4. Tolerance of *CaPOA*1-OX Arabidopsis plants to high salinity and osmotic stresses

To investigate whether *CaPOA1* overexpression enhances salt stress tolerance in Arabidopsis plants, we tested the effect of NaCl on the germination of *CaPOA1*-OX seeds. There was no difference in seed germination between wild-type (WT) and *CaPOA1*-OX seeds under normal conditions. In the presence of 500 mM NaCl, germination of wild-type and *CaPOA1*-OX seeds was significantly inhibited; however, the *CaPOA1*-OX seeds germinated better than the wild-type seeds. About 70-96 % of the seeds of *CaPOA1*-OX lines germinated on the MS medium containing 500 mM NaCl, whereas only 10 % of the seeds of the WT plants. *CaPOA1*-OX line # 3 exhibited the highest *CaPOA1* transcriptional levels and total peroxidase activity (Figure 4A and B). More importantly, the transgenic line # 3 germinated to the highest rate, compared to other transgenic lines such as # 2 and # 4 (Figure 5A). This indicates that *CaPOA1* overexpression in Arabidopsis plants increased NaCl tolerance during seed germination.

The loss-of-function assay showed that leaf discs of *CaPOA1*-silencing pepper plants exhibited reduced tolerance to high salinity stress. For the gain-of-function assays, we tested whether overexpression of *CaPOA1* confers enhanced tolerance in Arabidopsis seedling plants to NaCl treatment. Seven-day-old WT and *CaPOA1*-OX seedling plants were floated in the MS medium containing increasing concentrations of NaCl. As shown in Figure 5B, no growth difference were observed between WT and *CaPOA1*-OX seedling plants under 0 and 150 mM NaCl treatment; however, growth of WT seedling plants was more strongly inhibited by 175 mM NaCl compared to *CaPOA1*-OX plants. Both WT and CaPOA1-OX seedling plants failed to survive and grow in the MS media containing 200 mM NaCl. Determination of total chlorophyll content revealed a significantly higher loss of chlorophyll

pigment in WT plants in response to treatment with 175 mM NaCl compared to *CaPOA1*-OX plants. Furthermore, lipid peroxidation levels in *CaPOA1*-OX plants were significantly decreased after treatment with 175 mM NaCl, compared with WT plants, indicating that overexpression of *CaPOA1* enhanced the tolerance of Arabidopsis plants to high salinity stress.

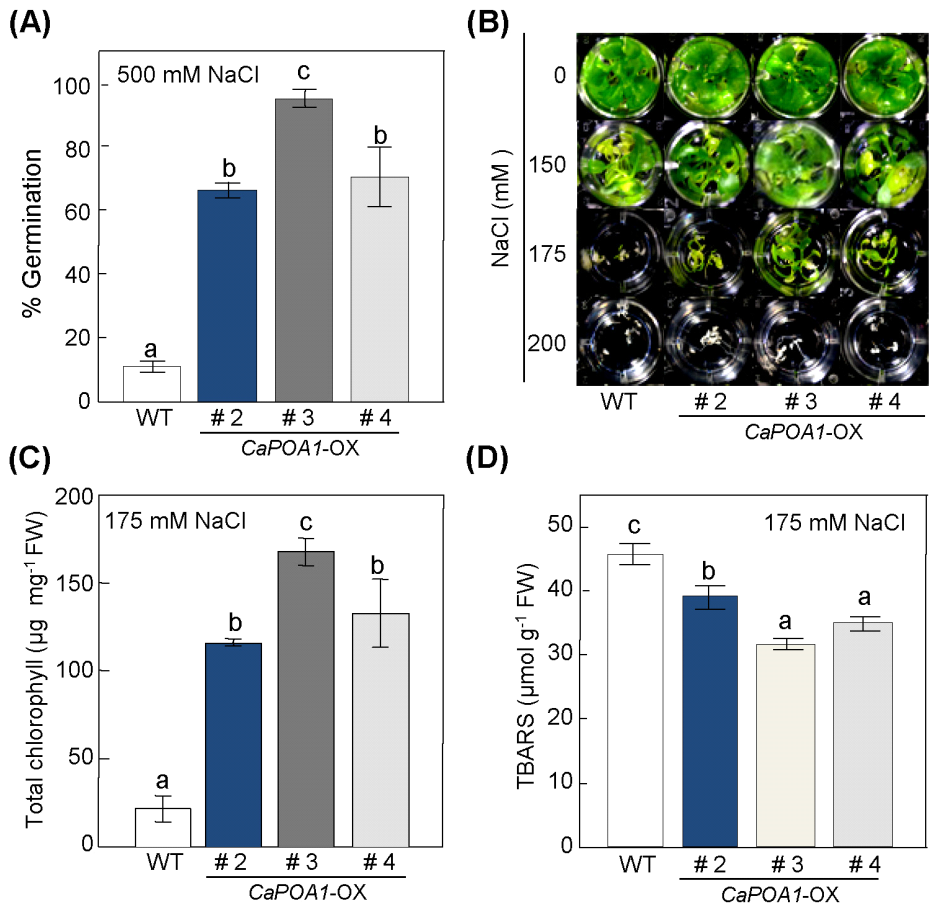

Figure 5. Enhanced tolerance of *CaPOA1*-OX seedling plants to salinity stress. (A) Enhanced germination of transgenic plant seeds on the MS medium containing 500 mM NaCl. (B) Representative photographs showed phenotypic differences between WT and *CaPOA1*-OX seedling plants treated with different concentrations of NaCl. (C) Chlorophyll content and (D) lipid peroxidation levels of WT and *CaPOA1*-OX seedling plants 7 days after treatment with 175 mM NaCl. Data are the means ± standard deviations from three independent experiments. Different letters indicate significant differences, as determined by the least significant difference test (P = 0.05).

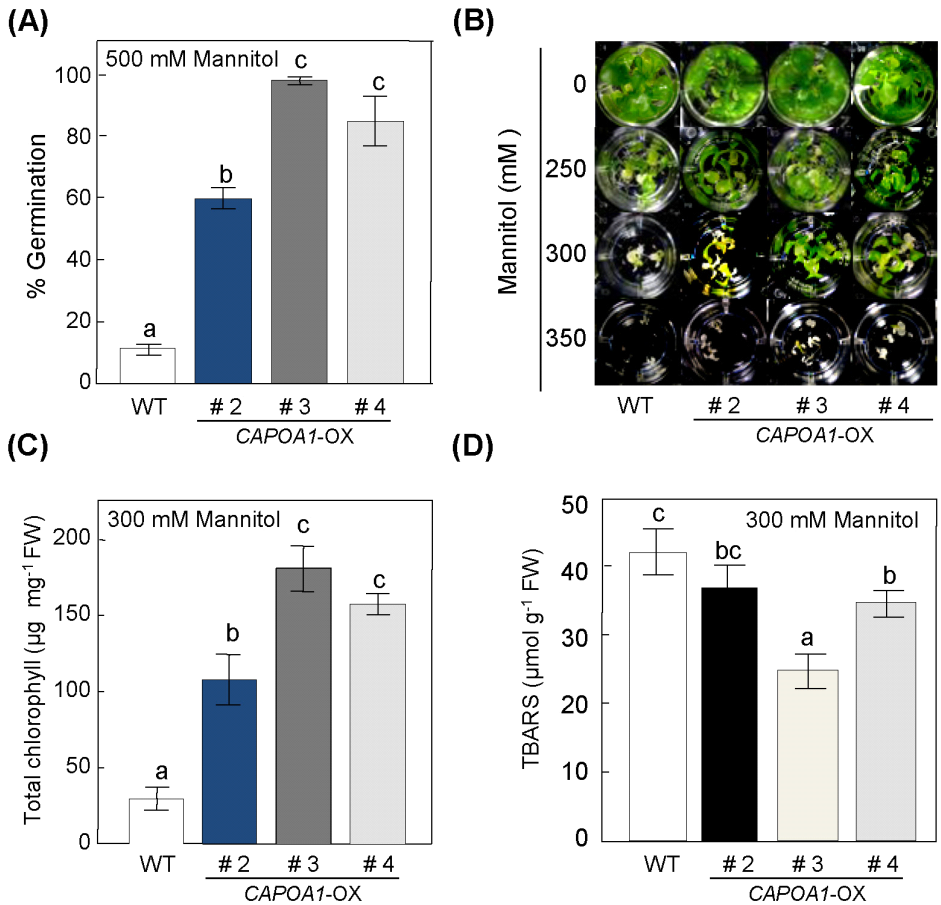

Figure 6. Enhanced tolerance of *CaPOA1*-OX seedling plants to osmotic stress. (A) Enhanced germination of transgenic plant seeds on the MS medium containing 500 mM mannitol. (B) Representative photographs showed phenotypic differences between WT and *CaPOA1*-OX seedling plants treated with various concentrations of mannitol. (C) Chlorophyll content and (D) lipid peroxidation levels of WT and *CaPOA1*-OX seedling plants 7 days after treatment with 300 mM mannitol. Data are the means ± standard deviations from three independent experiments. Different letters indicate significant differences, as determined by the least significant difference test (P = 0.05).

Overexpression of *CaPOA1* in Arabidopsis plants enhanced tolerance to osmotic stress caused by increasing mannitol concentrations (Figure 6). The germination rate of *CaPOA1*-OX seeds were significantly higher than that of WT seeds on the MS medium containing 500 mM NaCl (Figure 6A). No visible difference in growth between WT and *CaPOA1*-OX transgenic plants were observed under normal conditions

without NaCl (Figure 6B). However, treatment with gradually increasing concentrations of mannitol enhanced the tolerance of *CaPOA1*-OX seedling plants to osmotic stress compared to WT seedlings (Figure 6B). The chlorophyll content of *CaPOA1*-OX seedling plants grown in 300 mM mannitol was significantly higher than that of WT plants (Figure 6C). The level of lipid peroxidation at 300 mM mannitol was also significantly lowered in *CaPOA1*-OX transgenic plants compared to WT plants. These findings support the notion that transgenic expression of *CaPOA1* enhanced the tolerance to high salinity and osmotic stress in Arabidopsis plants.

5. Enhanced tolerance of *CaPOA1*-OX Arabidopsis plants to MV-induced oxidative stress

We tested the germination, chlorophyll content and lipid peroxidation levels of WT and *CaPOA1*-OX transgenic Arabidopsis seedlings after growing in the MS media containing increasing concentrations of MV (Figure 7). The *CaPOA1*-OX transgenic lines were highly tolerant to oxidative stress caused by 1 μM MV, which significantly inhibited the germination of WT seeds. Only approximately 10 % of WT seeds germinated at 1 μM MV; however more than 50 % of *CaPOA1*-OX seeds germinated (Figure 7A). Notably, the higher transcription and total peroxidase levels of the *CaPOA1*-OX line # 3 results in the higher tolerance to MV-induced oxidative stress than other transgenic lines # 2 and # 4. Transgenic seedling plants were markedly tolerant to gradually increasing concentrations of MV (Figure 7B). Treatment with 0.5 and 0.75 μM MV severely inhibited the growth of WT and *CaPOA1*-OX transgenic seedling plants; however, the growth of transgenic seedlings was inhibited to a lesser extent. In the presence of 0.5 μM MV, *CaPOA1*-OX plants retained significantly higher chlorophyll content compared to WT plants (Figure 7C). Furthermore, MV-induced

cell damage was also significantly reduced in transgenic plants, as observed by measuring TRABS content (Figure 7D). This suggests that overexpression of *CaPOA1* in Arabidopsis enhanced oxidative stress tolerance by enhancing total peroxidase activity.

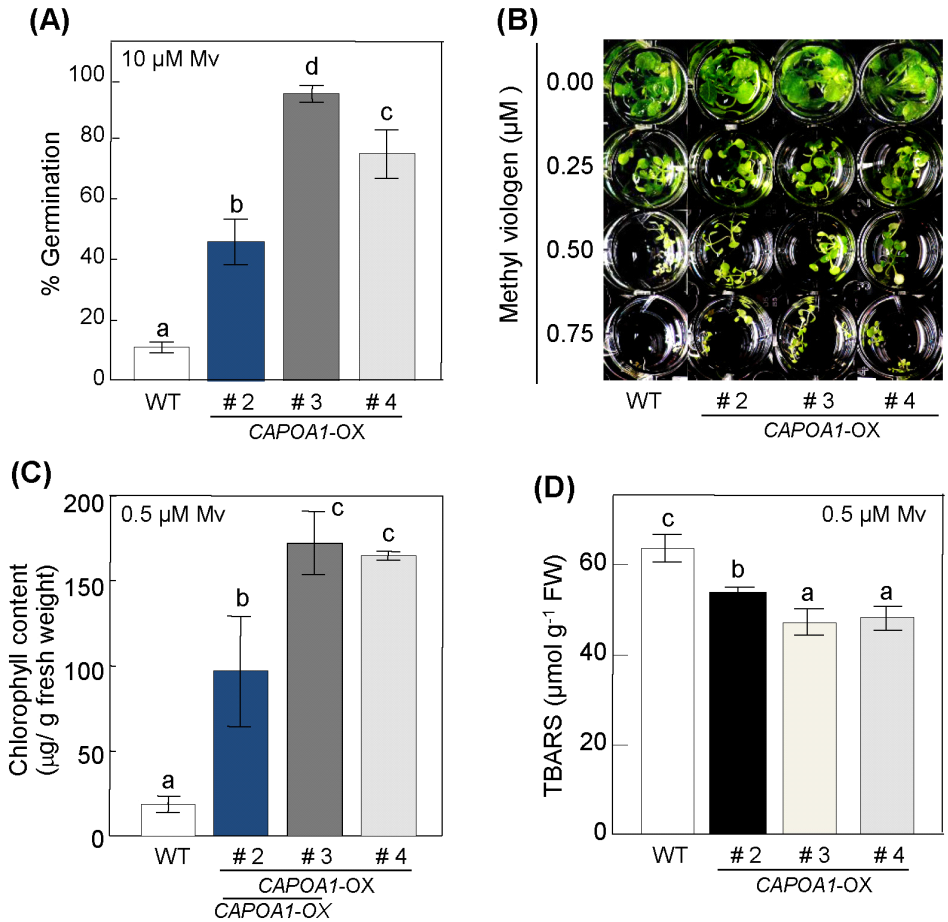

Figure 7. Enhanced tolerance of *CaPOA1*-OX seedling plants to methyl viologen (MV)-induced oxidative stress. (A) Enhanced germination of transgenic plant seeds on the MS media containing 10 μM MV. (B) Representative photographs showed phenotypic differences between WT and *CaPOA1*-OX seedling plants treated with various concentrations of MV. (C) Chlorophyll content and (D) lipid peroxidation levels of WT and *CaPOA1*-OX seedling plants 7 days after treatment with 0.5 μM MV. Data are the means ± standard deviations from three independent experiments. Different letters indicate significant differences, as determined by the least significant difference test (P = 0.05).

Arabidopsis thaliana is widely used as a model plant, because it is useful for plant molecular genetic experiments (Koornneef and Meinke, 2010). To further understand the function of CaPOA1, a phylogenetic analysis was performed based on the amino acid sequences of CaPOA1 and Arabidopsis ascorbate peroxidases (APXs) (Figure 8). Of the six different Arabidopsis APXs compared, APX3 had the highest sequence homology (66%) with CaPOA1. Thus, further comparative analyses of CaPOA1 and Arabidopsis APX3 will be interesting to understand whether these APXs have similar or distinct functions in abiotic stress tolerance and root development.

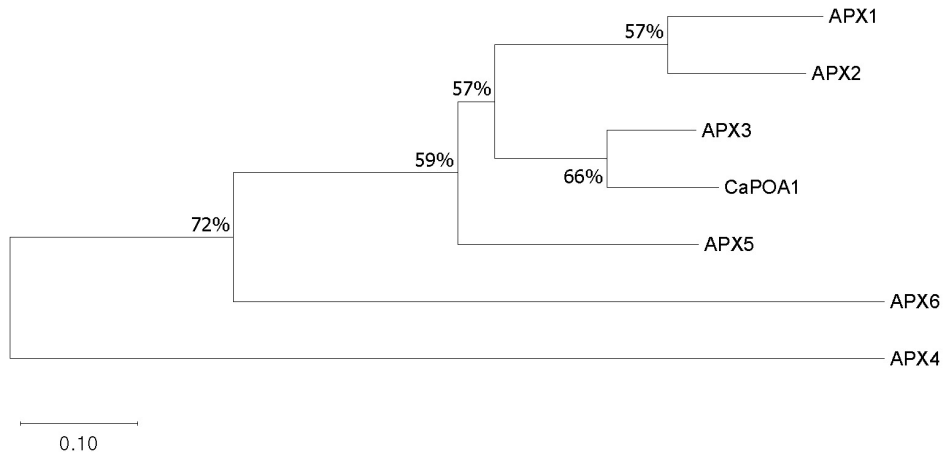

Figure 8. Phylogenetic tree analysis of *CaPOA1* with Arabidopsis APXs. CaPOA1 shows the highest sequence homology with Arabidopsis APX3 at the amino acid level. The phylogenetic tree was generated by MEGA X version 10.2.5 using the neighbor-joining method with a bootstrap replica count of 500.

IV. Discussion

Our results obtained in this study demonstrate that the expression of *CaPOA1* is not only induced by different abiotic stresses, but also plays an important role for detoxification of ROS generated during

oxidative stress. *CaPOA1*-silenced and *CaPOA1*-OX plants, respectively, had significantly reduced and enhanced tolerance to the various abiotic stresses tested in this study. These results suggest a protective function of *CaPOA1* during abiotic stress-induced oxidative bursts. Following the paradigm that plant ascorbate peroxidases (APXs) detoxify H_2O_2 into H_2O (Caverzan et al., 2012), we postulate that altered expression of *CaPOA1* affects the sensitivity of plants against different abiotic stresses. Thus, lipid peroxidation levels were enhanced and decreased in *CaPOA1*-silenced and *CaPOA1*-OX plants, respectively, leading to different sensitivities to high salinity, osmolality and MV-induced oxidative stress. These results collectively suggest that *CaPOA1* activity alleviates oxidative damage caused by abiotic stress, thereby improving plant survival.

One of the most obvious physiological responses of plants to various abiotic stress conditions is increased production of ROS (Pandey et al., 2017; Huang et al., 2019). In previous studies, transgenic overexpression of the APX gene improved ROS scavenging potential, thus leading to enhanced tolerance of different plants to various abiotic stresses (Singh et al., 2014; Pandey et al., 2017; Gorripati et al. 2021). Overexpression of *APX* in rice plants significantly enhanced drought stress tolerance at different stages of plant development, such as germination, seedling and reproductive stages (Gorripati et al., 2021). Transgenic rice plants overexpressing *APX* under drought stress conditions induced by 200 mM mannitol exhibited enhanced chlorophyll, proline and reducing sugar content as well as enhanced catalase superoxide dismutase activity. In contrast, drought stress induced malondialdehyde (MDA) production by lipid peroxidation. However, MDA production was significantly reduced in transgenic rice plants. Ectopic overexpression of *Salicornia brachiata peroxisomal APX (SbpAPX)* in peanut plants significantly enhanced saline stress tolerance (Singh et al., 2014). These findings in other plant *APXs* suggest that

CaPOA1 may be available as a useful genetic resource to enhance abiotic stress tolerance in different plant species.

Unexpectedly, *CaPOA1*-OX transgenic Arabidopsis plants showed a similar growth phenotype in above-ground parts; however they showed significantly reduced root growth and development compared to WT Aabidopsisr plants, In particular, *CaPOA1* overexpression in Arabidopsis reduced growth of primary roots and number of lateral roots. In Arabidopsis plants, 6 different APXs (APX1 to APX6) were identified from the genome sequencing data. However, very limited information is available for the role of APXs in root growth and development. Of the six different APXs from Arabidopsis, only APX1 was identified as an antioxidant enzyme regulating root growth and development (Correa-Aragunde et al., 2013). APX1 targeted auxin-induced denitrosylation in roots to induce suppression of auxin-induced root growth. The S-nitrosylated form of recombinant APX1 expressed in *Escherichia coli* was more active than the denitrosylated form, suggesting that auxin-induced denitrosylation by APX1 suppresses its H_2O_2 scavenging activity. In addition, Arabidopsis *apx1* mutants exhibited increased H_2O_2 accumulation in roots, shorter roots, and less sensitivity to auxin than the WT plants. However, in our study, the enhanced total peroxidase activity in *CaPOA1*-OX Arabidopsis plants reduced root growth of Arabidopsis, unlike previous studies of APX1 in Arabidopsis (Correa-Aragunde et al., 2013). *CaPOA1* overexpression may negatively regulate APX1 activity in Arabidopsis to inhibit root development in an unknown way.

In addition, phylogenetic analysis shows that *CaPOA1* shares the highest similarity with Arabidopsis APX3 (Figure 8). However, the role of APX3 in root development is not yet understood (Narendra et al., 2006). ROS are produced with various cellular oxidases in various cellular compartments such as chloroplasts, mitochondria, plasma membrane, peroxisomes, apoplasts, endoplasmic reticulum and cell wall (Van Breusegem and Dat, 2006; Sharma et al., 2012). ROS play an

important role in cells, acting as signaling molecules that regulate normal plant growth and also induce irreversible damage and cell death responses to environmental stresses. Tsukagoshi (2016) reported that levels of ROS regulate cell division of the root apical meristem, the transition from cell proliferation to cell differentiation, and root hair and lateral root development. For example, Root Hair Defective 2 (RHD2) mutants, which lack the Respiratory burst oxidase homolog protein C (RbohC) or NADPH oxidase genes, did not accumulate ROS and failed to develop early root hairs (Foreman et al., 2003). In addition, treatment of Arabidopsis plants with diphenylene iodonium (DPI), an inhibitor of NADPH oxidase activity, inhibited ROS accumulation and root hair development. In contrast, double mutations in the other NADPH oxidase genes, RbohD and RbohF, increased lateral root density and peroxidase activity in roots (Li et al., 2015). These findings suggest that overexpression of *CaPOA1* may induce an imbalance in ROS production in roots, thereby altering the growth and development of transgenic Arabidopsis roots. Therefore, further studies of the roles of different APXs and ROS on root development are needed to understand the underlying mechanisms of reduced root growth due to *CaPOA1* overexpression.

In summary, we investigated *in planta* function of *CaPOA1* during abiotic stress responses using *CaPOA1*-silenced and overexpression transgenic plants. All abiotic stresses tested in pepper plants induced the expression of *CaPOA1* and its silencing significantly reduced gene expression and tolerance of pepper plants to abiotic stresses. In contrast, overexpression of *CaPOA1* transformed in Arabidopsis significantly enhanced abiotic stress tolerance. Taken together, our results strongly suggest that high salinity, osmotic stress, and MV-induced oxidative stress trigger the expression of *CaPOA1*, leading to H_2O_2 scavenging and oxidative protection of pepper plants.

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