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Full Length Research Paper

Thylakoid-bound ascorbate peroxidase increases resistance to salt stress and drought in *Brassica napus*

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Reactive oxygen species (ROS) are cellular indicators of stress. In plants, they function as secondary messengers in response to environmental stress. Ascorbate peroxidase (APX) is an important enzyme directly involved in the scavenging of ROS. In this study, we aimed at identifying the function of the *Brassica napus* thylakoid APX (tAPX). Germination efficiencies of seeds of *B. napus* plants over expressing tAPX were higher than those of the seeds of the control plants; this was true both on Murashige and Skoog medium with 300 mM mannitol and with 150 mM NaCl. Further experiments showed that 40-day-old seedlings of the control plants turned yellow, withered, and subsequently died, when treated with 150 mM NaCl for 12 days. In contrast, transgenic plants over expressing tAPX survived this treatment and had at least three green leaves at the end of the experiment. When 40-day-old seedlings were withheld water for 10 days, followed by a 2 day recovery, the control plants exhibited smaller leaves and shorter stems in comparison to tAPX-over expressing plants. In addition, compared with control plants, tAPX-overexpressing plants show reduced hydrogen peroxide accumulation and increased APX relative activity. Our results demonstrate that tAPX plays an important role in resistance to salt stress and drought in plants.

Key words: tAPX, transgenic lines, *Brassica napus*, salt stress, water deficiency.

INTRODUCTION

Reactive oxygen species (ROS), such as O_2^-, H_2O_2 and -OH, are produced throughout plant development and in response to biotic and abiotic stresses. They participate in cellular signaling, but also have damaging effects on membrane lipids, proteins, chlorophyll and nucleic acids. Plants have developed an efficient antioxidant defense system to cope with ROS (Foyer and Noctor, 2005). In *Arabidopsis*, at least 152 genes are involved in managing the levels of ROS (Mittler et al., 2004). One of the most efficient ways for plants to maintain the balance between ROS production and scavenging is to regulate the activity of antioxidant enzymes, including superoxide dismutases, ascorbate peroxidases (APXs), glutathione reductases and catalases (Yan et al., 2003).

APX reduces hydrogen peroxide (H_2O_2) to water and hence scavenges ROS directly (Asada, 1999). Previous studies indicated that different APXs are localized in the mitochondria (De Leonardis et al., 2000), chloroplasts (Jespersen et al., 1997), peroxisomes (Zhang et al., 1997) and cytosol (Caldwell et al., 1998). In chloroplasts, there are at least three different APX isozymes: thylakoid-bound APX (tAPX), lumen APX and stromal APX. Previous results demonstrated that APX contributes to ROS scavenging and to ROS signaling (Maruta et al., 2010). Over expression of tAPX increased the resistance of *Arabidopsis* plants to paraquat-induced photooxidative stress and nitric oxide-induced cell death (Murgia et al., 2004). Moreover, expression of the *Cyanidioschyzon merolae* sAPX in *Arabidopsis* enhanced thermotolerance in the receiving plants (Hirooka et al., 2009), and overexpression of the tomato *tAPX* gene in tobacco improved tolerance to high- or low-temperature stress in these plants (Sun et al., 2010). Down regulation of *tAPX* in *Arabidopsis* enhanced paraquat-induced photo-

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Abbreviations: **ROS**, Reactive oxygen species; **APX**, ascorbate peroxidases; **tAPX**, thylakoid-bound ascorbate peroxidase; **MS**, Murashige and Skoog (1962); **DAB**, 3,3-diaminobenzidine; **AsA**, ascorbic acid.

oxidative stress and nitric oxide-induced cell death (Tarantino et al., 2005). On the other hand, other findings suggest that chloroplast APX enzymes may not be so crucial for H_2O_2 scavenging, or that other antioxidative systems can compensate for chloroplast APXs (Davletova et al., 2005). Therefore, the exact role of tAPX in plant stress resistance still needs further clarification.

The *tAPX* gene (GenBank accession no, FJ965556) used in this study was cloned from *Brassica napus* L. Its amino acid sequence identity scores with *Brassica oleracea* and *Arabidopsis thaliana* were 95% and 77%, respectively (Liu et al., 2010). Prediction of the protein sequence of tAPX reveals that there is a transit peptide for chloroplast localization using TargetP 1.1. In order to study the functions of tAPX, we successfully generated tAPX-overexpressing *B. napus* lines using *Agrobacterium tumefaciens*-mediated transformation. RT-PCR was performed to analyze transcript levels of tAPX. We compared the transgenic lines with control plants under salt stress or drought. Our results demonstrated that tAPX functions in the resistance of plants to salt stress and water deficiency by reducing the accumulation of H_2O_2.

MATERIALS AND METHODS

Plant materials

Three independent *B. napus* transformant lines (21-7, 3-10 and 15-10) and controls (ck) were used in the experiments. All plants were grown under normal growth conditions (24°C day/16°C night, 16-h photoperiod), with around 70% relative humidity. Seeds were sterilized in 70% EtOH for 30 s, washed several times in distilled sterile water, soaked in 0.1% HgCl for 10 min, and rinsed another 5–6 times. They were then planted on MS medium with or without the supplement of NaCl or mannitol. Plates were incubated under the same growth conditions described above. Young seedlings were used for transformation or transferred to pots.

DNA transformation and identification of transgenic lines

tAPX was amplified from cDNA of young *B. napus* seedling leaves, and cloned into pBI121. *A. tumefaciens*-mediated transformation was performed as described by Wu et al. (2009). Hypocotyls, derived from 7-day-old seedlings, were precultured for 2 days, co-cultured with *A. tumefaciens* containing pBI121-tAPX for 2 days, and then transferred to MS medium containing 10 mg/l kanamycin and 500 mg/l carbenicillin. The co-cultured hypocotyls were subcultured every 10 days in fresh medium of the same composition. The first small shoots appeared after 3–4 weeks. Healthy shoots were transferred to half strength (1/2) MS medium. Rooted shoots were transferred to pots for seed production or for use in genomic PCR and RT-PCR analyses.

Genomic PCR was performed to verify whether the plants were successfully transformed. Genomic DNA was isolated from leaves according to Sambrook and Russell (2002). The following primer sets were used: CaMV 35S promoter specific (forward) primer (5′-TCCCCCCGGGGCAAGACCCTTCCTC-3′) in combination with tAPX2 (5′- CCGGAATTCTTAGAAACCAGAGTAATCAGAGGAGTT-3′). These primers generate a fragment of close to 1300 bp. The PCR program used for this procedure included 30 cycles at 94°C for 40 s, 58°C for 40 s, and 72°C for 1 min. All reactions were preceded by a primary denaturation step at 94°C for 4 min and a final extension at 72°C for 5 min. PCR products were separated on a 1.2% (w/v) agarose gel. A non-transgenic plant was used as negative control, while *A. tumefaciens* containing pBI121-tAPX was used as positive control.

RT-PCR analysis of transgenic plants

Total RNA was isolated from leaves of transgenic lines and control plants using the RNA extraction Kit (TIANGEN, P. R. China). The RT-PCR was performed using the RevertAid first strand cDNA synthesis kit (MBI Fermentas, Canada), according to the manufacturer's instructions. RT-PCR was used to determine *tAPX* transcript levels in tAPX-overexpressing lines and control plants. An approximately 470-bp *tAPX* fragment was amplified with the following primers: tAPXup, 5′-ACAGAGAGGTGGGGCTAACGG-3′ and tAPXdn, 5′-ACTTGAGCCATTTCACTGTCCAT-3′. The following primers, which amplify an approximately 400-bp fragment of β-actin, were used as the control primers: 5′-GTGGGGATG GAAGCTCCTG-3′ and 5′-GTGATCTCTTTGCTCATACGGTC-3′. The PCR program included 23 cycles at 94°C for 40 s, 60°C for 30 s, and 72°C for 40 s. Other parameters were the same as in the genomic PCR.

The relative activity of APX

Following Shen's (Shen et al., 1996) procedure, the relative activity of APX was measured by decrease of ascorbate at 290 nm due to ascorbate oxidation. Plants of 25-day old seedlings were used for test the relative activity of APX. Sample about 1g was grinded carefully with 5ml extracting solution (50mM PBS, pH7.8, 2mM AsA, 5mM EDTA, fresh prepared), the mix was centrifuged 20min at 4□ (10000×g), the supernatant was placed on ice for use. The same volume of supernatant of each sample was added to reaction solution (50mM PBS, pH7.0, 0.5 mM AsA, 0.1 mM EDTA, 0.1mM H_2O_2), absorbance at 290nm was recorded using TU-1901 spectrophotometer (Beijing Perkinje general instrument Co., Ltd), adding the sample to reaction solution has no H_2O_2 as control. Enzyme oxiodate 1 uM AsA at room temperature in one minute was looked as enzyme activity unit (U). Each experiment was repeated 3 times.

Salt stress and drought treatment

For seed germination assays, full seeds were selected and spread on MS medium supplemented with 150 mM NaCl or 300 mM mannitol. The seed germination rate was assessed after 5 days. A total of 30 seeds were used in each experiment, and each experiment was repeated 3 times.

Full seeds were germinated on wet filter paper under normal conditions, and were transferred to pots after 5 days. 40-day-old seedlings were tested for resistance to salt stress and drought. All plants, transgenic lines and controls were divided into four sets. The first set was watered with 1/2 MS containing 150 mM NaCl once every other day for 12 days. The second set received 1/2 MS only and served as the control. The third set was not watered at all for 10 days, and was then given time to recover for 2 days. The last set received plenty of water, and served as the control for the drought-treated plants. All plants grew under normal conditions.

H_2O_2 staining with DAB

H_2O_2 was visualized in leaves using 3,3-diaminobenzidine (DAB) as the substrate. Following Murgia's description (Murgia et al., 2004), *B. napus* leaves were cut and submerged in 5–6 ml of 1 mg/ml DAB

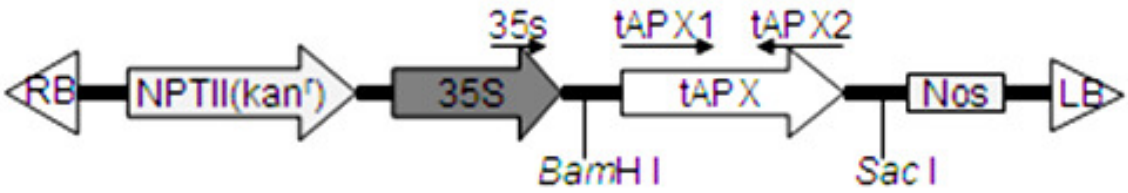

Figure 1. tAPX overexpression vector. The BamHI-SacI fragment of the *B. napus* tAPX cDNA was inserted into the pBI121 vector. The position of primers, used for confirming the transgenic lines, is indicated by arrows.

solution (Amresco, USA) in culture dishes. Petioles were cut again once fully immersed in order to prevent cavitation. The leaves were incubated in the solution overnight in the dark. The next day, leaves were rinsed twice in distilled water and transferred to Falcon tubes containing 96% EtOH. They were subsequently placed in boiling water until the chlorophyll was completely removed from the leaves (about 15–20 min). Leaves were then transferred to fresh 96% EtOH.

RESULTS

Production and identification of *B. napus* lines overexpressing tAPX

A. tumefaciens EHA105 was chemically transformed with pBI121-tAPX (Figure 1). For plant transformation, *B. napus* hypocotyls were used. After the transformation procedure was completed, 52 regenerated *B. napus* seedlings were transferred to pots. In order to identify the transgenic lines, genomic PCR was performed, using leaf genomic DNA. Nine independent transgenic lines were identified (Figure 2). Of these, three lines (21-7, 3-10 and 15-10) were selected for subsequent analyses.

RT-PCR analysis of *B. napus* lines overexpressing tAPX

tAPX transcript levels were analyzed in tAPX-over-expressing lines and controls by RT-PCR amplification of a *tAPX* fragment. β-Actin transcript was amplified as the control. The results indicate that in tAPX-overexpressing lines, *tAPX* mRNA accumulated to higher levels than in the control plants (Figure 3). The lines 3-10 and 15-10 showed a 3- to 5-fold increase in *tAPX* mRNA, while in 21-7, the *tAPX* mRNA level was 10 times higher than that in the control.

Transgenic lines overexpression tAPX show higher APX activity

Whole plant of 25-day old seedlings including three transgenic lines and control plants were used for testing the relative activity of APX. The results show that all of 3 transgenic lines have higher APX activity than control plants. In contrast with control plants, line 21-7, 3-17, 15-10 has more 37%, 27%, 18% APX activity, respectively (Figure 4). This result is highly consistent with the fact that the higher tapX transcript levels and lower H_2O_2 accumulation in transgenic lines in comparison to control plants. We conclude that the overexpressing tAPX in *B. napus* contributes the higher APX relative activity in transgenic lines.

Overexpression of tAPX results in lower levels of H_2O_2

H_2O_2 was visualized in leaves using DAB as the substrate. Higher concentrations of H_2O_2 result in more intense DAB staining. To examine the role of tAPX in scavenging H_2O_2, leaves of tAPX-overexpressing lines and control plants were treated with DAB. The results show that DAB staining in the leaves of the two control plants was intense, while the leaves of the three transgenic lines were less intensely stained. Only the basal part of the leaves, petioles, and main veins were dark. It means that the tAPX-overexpressing plants has reduced H_2O_2 accumulation compared to the control plants (Figure 5). In line 21-7, DAB staining was the weakest, indicating that the H_2O_2 level was lowest in these plants. The results suggest that the tAPX-overexpressing plants had higher H_2O_2 scavenging capacity than did the control plants. This is consistent with the fact that the highest level of *tAPX* transcript was detected in line 21-7, the line with the weakest DAB staining.

tAPX overexpression increased germination efficiency on exposure to mannitol and NaCl

All seeds were sterilized and planted on MS medium supplemented with 300 mM mannitol or 150 mM NaCl, and cultured under normal growth condition. The germination rate was assessed after 5 days. The data indicated that the three tAPX-overexpressing lines showed higher seed germination rates than did the controls (Figure 6). Under

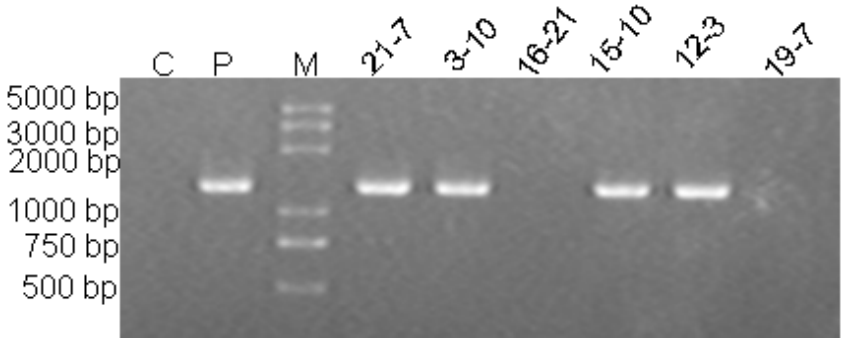

Figure 2. Genomic PCR analysis of *B. napus* plants overexpressing tAPX. C, control; p, positive control; m, marker (molecular sizes are shown on the left); 21-7, 3-10, 15-10, and 12-3, transgenic lines; 16-21 and 19-7, plants in which transformation was unsuccessful.

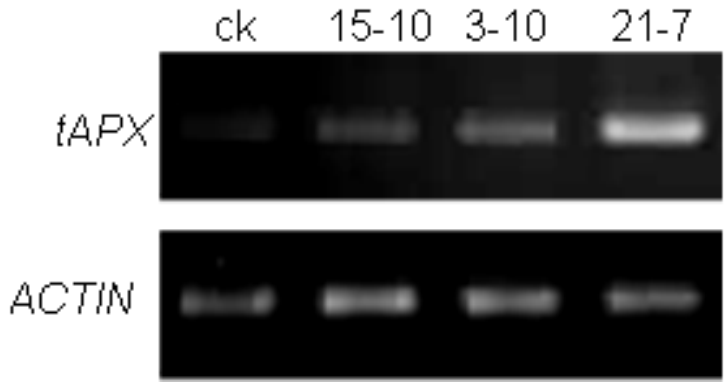

Figure 3. RT-PCR analysis of tAPX-overexpressing and control plants. 15-10, 3-10, and 21-7, tAPX-overexpressing lines; ck, control. Amplification of β-actin serves to verify that equal amounts of RNA were present in each sample.

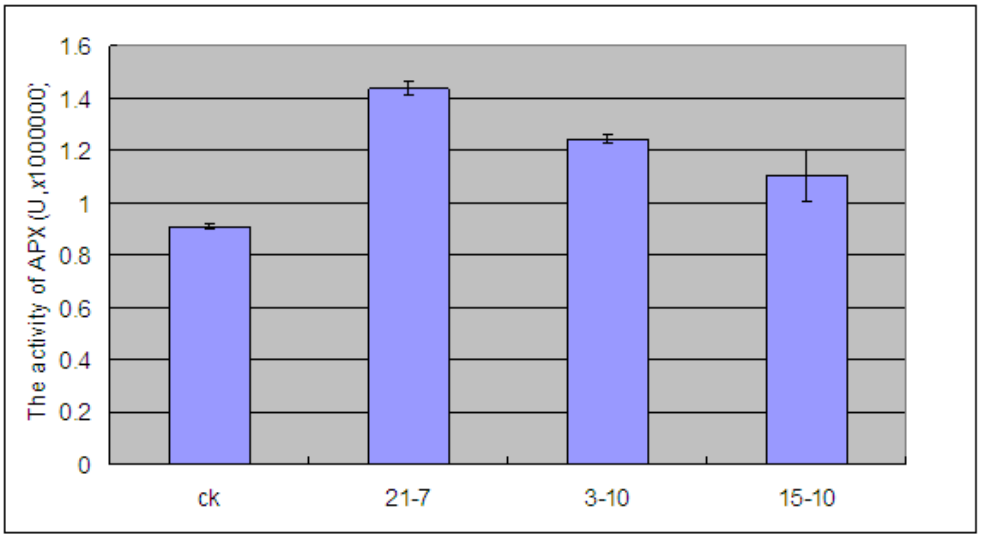

Fig. 4 The relative activity of APX. The tAPX-overexpressing lines (21-7, 3-10, and 15-10) show higher APX relative activity than the control plants (ck).

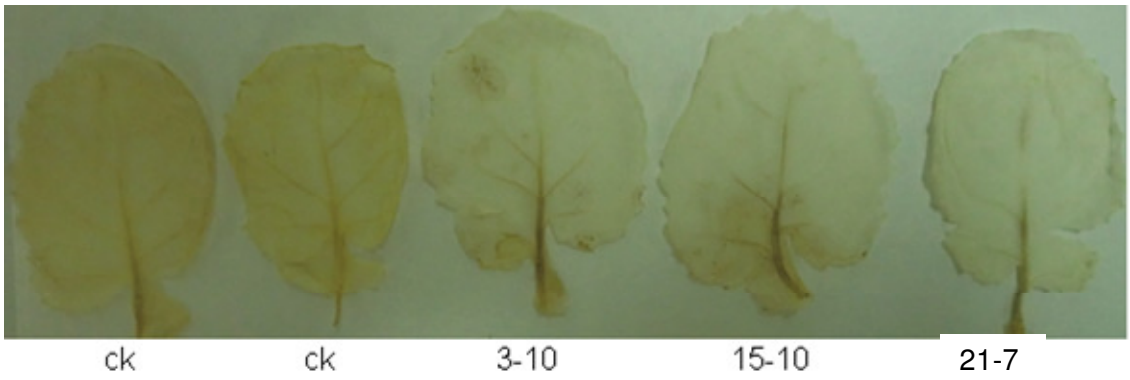

Figure 5. DAB staining of leaves. tAPX-overexpressing lines (3-10, 15-10, and 21-7) accumulate H_2O_2 to lower levels than did the control plants (ck).

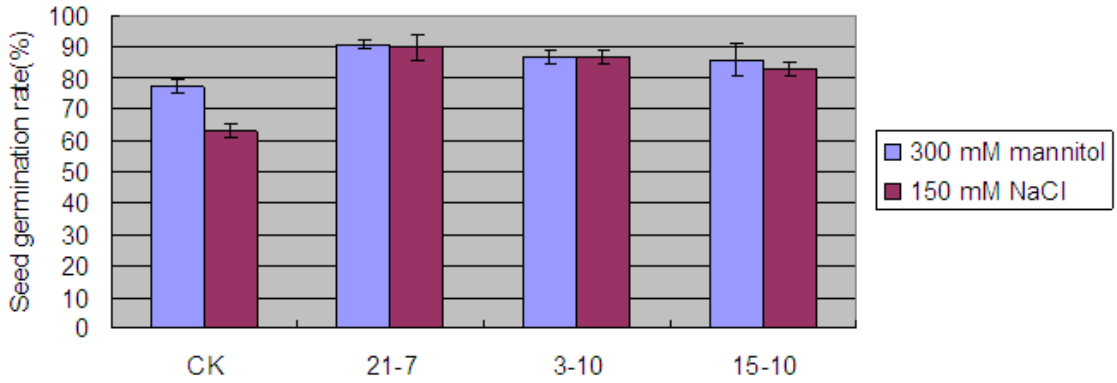

Figure 6. Seed germination rates under salt and osmotic stress. Seed germination is more efficient in tAPX-overexpressing lines under salt and osmotic stress. 21-7, 3-10, and 15-10, tAPX-overexpressing lines; ck, control. Results represent the mean of three experiments. Bars correspond to the standard deviation.

mannitol stress, the lowest and highest seed germination rates of the tAPX-overexpressing lines were 86% (15-10) and 91% (21-7), respectively, while the germination rate of the control was 77%. On NaCl, the seed germination rate of the control seeds was 63%, while the tAPX-overexpressing lines reached germination rates ranging from 83 to 90%. The results showed that the tAPX-overexpressing lines had developed increased resistance to salinity and osmotic stress at the seed germination stage.

Seedlings of tAPX-overexpressing lines increase resistance to NaCl stress and drought

When watered with 1/2 MS supplemented with 150 mM NaCl, or not watered at all, all plants including the tAPX-overexpressing lines were affected (Figure 7B and D). Most plants had only three to four leaves left; no new leaves were produced and old leaves turned yellow, withered, and fell off. However, tAPX-overexpressing lines showed a certain degree of resistance to the stress. When subjected to salt stress, specific differences between control and transgenic plants were observed. The control plants withered and died while the transgenic plants were still alive bearing three to four green leaves (Figure 7B). This means that tAPX-overexpressing lines displayed a high degree of resistance to salt stress.

When plants were subjected to 10 days of water deficiency, followed by 2 days of recovery, we again observed different symptoms in the control and transgenic plants. Compared with transgenic lines, the damage to control plants appeared more severe (Figure 7D). These observations demonstrated that the tAPX-overexpressing lines showed increased resistance to drought.

DISCUSSION

Plant cells produce ROS in many processes associated with plant growth and development. ROS function as second messengers and their accumulation is crucial to plant development(Foyer and Noctor, 2005). On the other

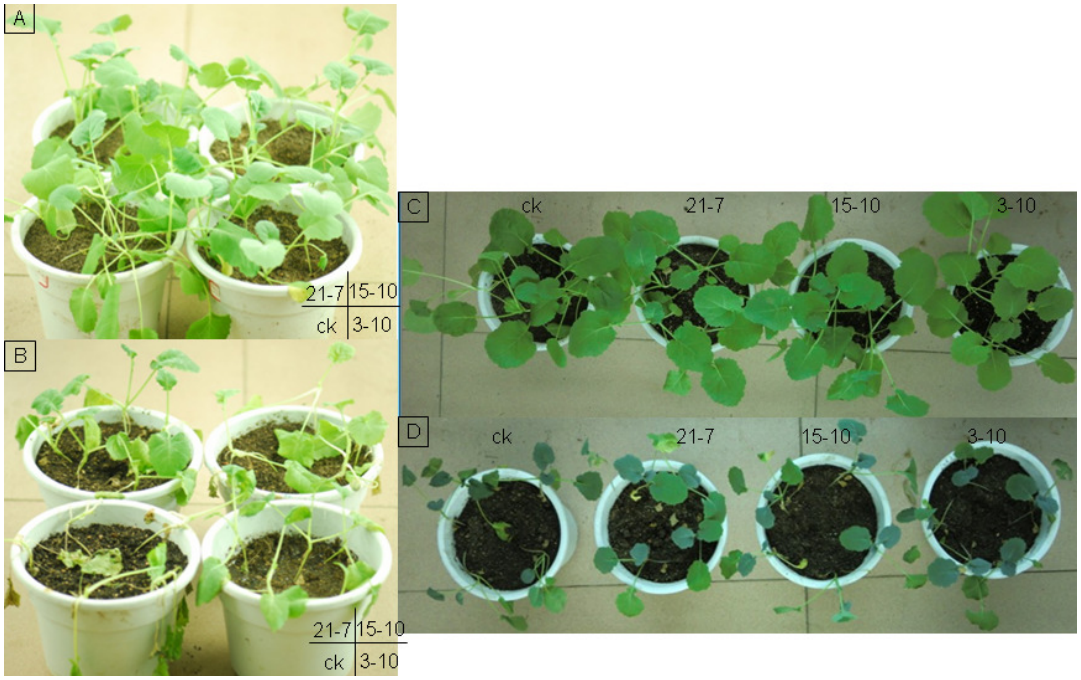

Figure 7. Seedlings of tAPX-overexpressing lines showing increased resistance to salt stress (B) and water deficiency (D). A and C; 52-day-old seedlings cultivated under normal growth conditions. B, 40-day-old seedlings treated with 1/2 MS supplemented with 150 mM NaCl for 12 days, once every other day. D, Drought-treated 40-day-old seedlings (10 days drought treatment and 2-day recovery). Controls (ck) and tAPX-overexpressing lines (21-7, 3-10, and 15-10) are labeled in the panels.

hand, high ROS concentrations damage membrane lipids, proteins, chlorophyll, and nucleic acids, and they trigger genetically programmed cell death. Therefore, ROS can be seen as cellular indicators of stress and key secondary messengers involved in the plant's response to environmental stresses. To cope with ROS, plants have developed efficient defense systems to maintain the balance between ROS production and scavenging. APX is one of the most important ROS scavenging enzymes in plants, reducing H_2O_2 to water using ascorbic acid (AsA) as the electron donor (Asada, 1999).

Overexpression of APX enzymes in different plant species revealed key roles of the enzymes in the protection of plants against various forms of stress (Kwon et al., 2002; Tang et al., 2006; Yan et al., 2003; Lee et al., 2007; Murgia et al., 2004; Hirooka et al., 2009; Sun et al, 2010). In order to examine the role of APX in ROS scavenging, APX knockout or knockdown mutants have been studied extensively. Antisense reduction of tAPX transcript levels in *Arabidopsis* enhanced paraquat-induced photooxidative stress and nitric oxide-induced cell death (Tarantino et al., 2005). On the other hand, some findings suggested that chlAPXs (tAPX and sAPX) are not crucial for H_2O_2 scavenging, or that other antioxidative systems can compensate for reduced chlAPX activity (Davletova et al., 2005). In order to clarify the role of tAPX in plant stress resistance, we generated and characterized tAPX-overexpressing *B. napus* plants, analyzed seed germination rates, and observed the resistance phenotypes of both transgenic lines and control plants under salinity and drought.

Salinity is a major abiotic stress, affecting agricultural production as a result of salt-induced oxidative damage, including high H_2O_2 production (Anjum et al., 2010). Salinity has a severe effect on seed germination in *B. napus*. In our experiments, seed germination rate on MS medium supplemented with 150 mM NaCl was highest in the transgenic lines (90%), while that of the control seeds was only 63%. Moreover, when 40-day-old seedlings were treated with 1/2 MS supplemented with 150 mM NaCl, the control plants withered and died, while the transgenic plants survived and had three to four green leaves. The results of this work indicated that the tAPX-overexpressing *B. napus* plants exhibit increased tolerance to salt stress.

Drought is likely to be the most common abiotic stress limiting crop yield worldwide. This form of stress also produces oxidative damage, including high H_2O_2 production. On MS medium supplemented with 300 mM mannitol, seed germination rate was highest in the transgenic lines (up to 91%), while that of the control seeds was 77%. The differences in seed germination rates between transgenic and control plants on mannitol medium were lesser than those on NaCl medium. When 40-day-old seedlings suffered from water deficiency after not been watered for 10 days, followed by 2 days of

recovery, the control plants had the smallest leaves and the shortest stems. We concluded that the tAPX-over-expressing *B. napus* plants show increased resistance to drought.

The leaves of transgenic and control plants were stained with DAB. The results show that leaves of tAPX-overexpressing plants has lower levels of H_2O_2 than did the control leaves. This is consistent with the resistance phenotypes under salinity and drought. We hypothesized that the high H_2O_2 scavenging capacity in the *B. napus* tAPX-overexpressing plants was due to the high level of tAPX; not to increased tAPX activity. Because AsA is mainly regenerated from monodehydroascorbate or dehydroascorbate during salt stress (Asada, 1999), the tAPX activity is maintained at the same level in the transgenic and control plants.

Based on those results, we concluded that the tAPX-overexpressing *B. napus* plants show increased resistance to salinity and drought. Although the stress treatments we used were not as severe as in those applied by others, our results are in accordance with their findings (Murgia et al., 2004; Hirooka et al., 2009; Sun et al., 2010). Summarily, in order to clarify the function of tAPX in plant growth and development, further experiments are required. For example, the phenotypes of transgenic plants under severe stress have to be compared to those in control plants and the gene expression of transgenic and control plants under such severe stress should be profiled.

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