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Full Length Research Paper

Ascorbate peroxidase gene from *Brassica napus* enhances salt and drought tolerances in *Arabidopsis thaliana*

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A full-length cDNA clone, *BnAPX* (GenBank: FJ965556.1) encoding ascorbate peroxidase and isolated from *Brassica napus*, was successfully introduced into *Arabidopsis thaliana*. Investigation into the function of *BnAPX* demonstrated that *BnAPX* transgenic plants grew better than wild type under NaCl stress, and also had higher germination rate and longer root length. Moreover, under the drought stress, *BnAPX* transgenic seeds displayed higher germination rate and the seedlings showed reduced wither and apoptosis phenomena. Therefore, our studies revealed that *BnAPX* was able to enhance the environmental tolerance of *Arabidopsis* to salt and drought stresses. In addition, transgenic *Arabidopsis* had lower H_2O_2 content because of the insertion and the overexpression of *BnAPX.* So, the mechanism of the tolerances in transgenic lines can be explained by reduction of H_2O_2 injury.

Key words: Ascorbate peroxidase, *Arabidopsis thaliana*, salt tolerance, drought tolerance.

INTRODUCTION

Soil salinity is a major abiotic stress to crops, and it is a main problem to agriculture yield on a global scale (Zhu, 2001). Salt stress inhibits the growth and development of higher plants and reduces their photosynthesis, respiration and protein synthesis. It can also disturb the nucleic acid metabolism further (Levine et al., 1990; Bray et al., 2000). Drought is another global problem to crop production, and it is reported that 50% of world rice production is influenced seriously by drought (Wani et al., 2010b; Bouman, et al. 2005). It is predictable that drought and salinity may cause serious salinization of more than50%of all arable lands by the year 2050 (Wang et al., 2003). Under salt stress, both ionic and osmotic balances are damaged in plants, which consequently result in the accumulation of reactive oxygen species (ROS) (Wang et al., 2003). ROS generated by exogenous stress such as salt stress and drought stress, damages membranes and macromolecules (Price et al., 1989; Moran et al., 1994) and injures plant cells or even the whole plants further. In addition, H_2O_2 as a non-polar molecule, can diffuse between different cellular compartments (Henzler and Steudle, 2000; Bienert et al., 2007), and it can accumulate in cells because of its stability. So, scavenging endogenous H_2O_2 timely can enhance plants' environmental tolerance to a certain degree.

Ascorbate peroxidase (APX, EC 1.11.1.11) catalyzes the reduction of H_2O_2 to water, using ascorbic acid (AsA) as the specific electron donor (Foyer and Hailiwell, 1976). It is a crucial enzyme scavenging H_2O_2 in the chloroplasts of plant cells (Asada, 1992). Its important physiological and biochemical functions have earned widespread attentions in recent years. *Arabidopsis* overexpressing thylakoidal ascorbate peroxidase (*tAPX*) was more resistant to photo-oxidative stress induced by Paraquat (Pq) than the wild type and reduced the symptoms of cell death induced by nitric oxide (Murgia et al., 2004), while

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Abbreviations: APX, Ascorbate peroxidase; **ASA,** ascorbate; **CTAB,** cetyltrimethylammonium bromide; **DAB,** 3, 3'-diaminobenzidine tetrahydrochloride; **H_2O_2,** hydrogen peroxide; **Kan,** kanamycin; **MS,** Murashige and Skoog medium; **PCR,** polymerase chain reaction; **ROS,** reactive oxygen species.

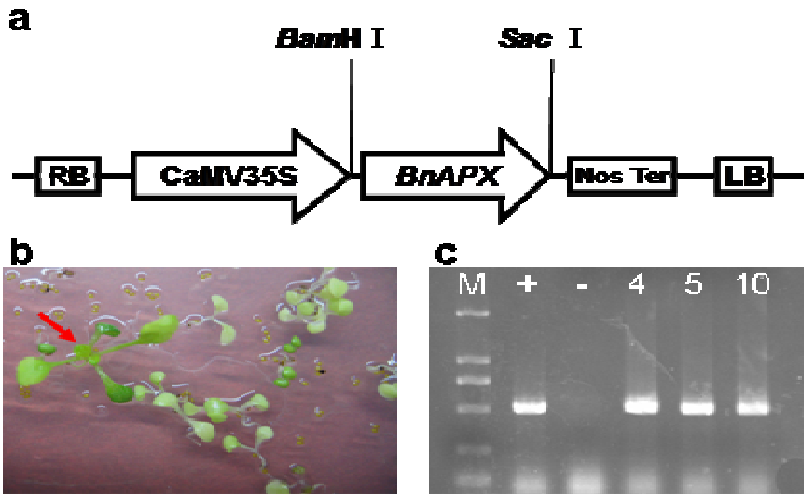

Figure 1. The identification of transgenic *Arabidopsis*. (a) Diagrammatic representation of the recombinant plasmid pCAMBIA-2301-BnAPX; (b) screening for transgenic *Arabidopsis* thaliana seedlings of T_0 generation transformed with pCAMBIA-2301-BnAPX vector on Kan medium; (c) the presence of the *BnAPX* sense sequence verified by PCR. M, DNA ladder DL 2000; +, *Agrobacterium* containing plasmid pCAMBIA-2301-*BnAPX* as the positive control; -, wild type as the negative control; 4, 5 and 10, three individual *BnAPX*-overexpressing lines.

Arabidopsis with suppressed expression of *tAPX* showed the opposite phenomena (Tarantino et al., 2005). Two kinds of rice cytosolic ascorbate peroxidases (*OsAPXa* and *OsAPXb*) could improve salt tolerance in transgenic *Arabidopsis* (Lu et al., 2007). The transgenic *Arabidopsis* overexpressing *HvAPX1*, a peroxisomal APX gene from barley also had increased tolerance to salt stress (Xu et al., 2008). A cytosolic APX from tomato could enhance its tolerance to chilling and salinity stresses (Wang et al., 2005). It was demonstrated that APX was able to enhance tolerances of plants to environmental stresses.

In our previous researches, *B. napus APX* gene (*BnAPX*) was cloned and sequenced. The amino acid sequence of the *BnAPX* had 77% homology to thylakoidal ascorbate peroxidase (*tAPX*) from *Arabidopsis*, and it was located in the chloroplast (Liu et al., 2010, 2011). In this article, *BnAPX* was introduced into *Arabidopsis* and overexpressed. Under the stress of different concentrations of NaCl, transgenic plants had longer root length but lower H_2O_2 content. The transgenic plants also grew better than wild type under drought stress. This is to say, the overexpressing lines were more tolerant to salt and drought stresses than wild type indeed. This result implies that *B. napus* could also survive in environment by transgenic technology, which would probably improve edible oil production.

MATERIALS AND METHODS

Plant material and growth conditions

Arabidopsis ecotype Columbia was used in this study. After three days of jarovization at 4°C, seeds of *Arabidopsis* were first surface sterilized in 75% ethanol for 1 min followed by immersion in 0.1% $HgCl_2$ for 10 min, and rinsed at least three times with sterile distilled water and then the seeds were germinated on MS solid medium (Murashige and Skoog, 1962) or in soils-mixture of vermiculite: peat (1:1) in climatic chamber at 22°C with 16/8 h of light/dark cycle and 70% humidity.

Transformation of *Arabidopsis*

The full length of *BnAPX* was cut from pMD18-BnAPX with B*am*H 1 and S*ac* 1 restriction sites and fused into the binary vector pCAMBIA-2301 in sense orientation under the control of the cauliflower mosaic virus 35S (CaMV35S) promoter (Figure 1a). The recombinant plasmid pCAMBIA-2301-BnAPX was first introduced into *Agrobacterium* strain EHA105, and then into *Arabidopsis* via *Agrobacterium*-mediated inflorescence-dip method (Clough and Bent, 1998). The transgenic seeds (T_0) were obtained and the seeds were then plated on MS medium with 50 mg/L kanamycin (Kan), and the Kan-resistant plants were transferred to the soil to produce the T_1 seeds by selfing. The homozygous lines (T_2) were then obtained from the selfed T_1 and confirmed by polymerase chain reaction (PCR) analysis.

Genomic DNA was extracted from three-week-old *Arabidopsis* with cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1990) and used as the template for the PCR analysis with specific primers. The presence of the *BnAPX* sense sequence in the selected plants was verified by PCR with the forward primer annealed to the CaMV 35S promoter of pCAMBI-2301 35Ssrx: (5′-CCTTCGCAAGACCCTTCCTC-3′) and the reverse primer annealed to the coding region (Zj: 5′- CCGTTAGCCCCACCTCTCTGTG -3′).

Transcriptional analysis of *BnAPX* by semi-quantitative PCR

Total RNA was prepared from three-week-old transgenic (T_2) and wild type seedlings using Trizol reagent (TIANGEN, China)

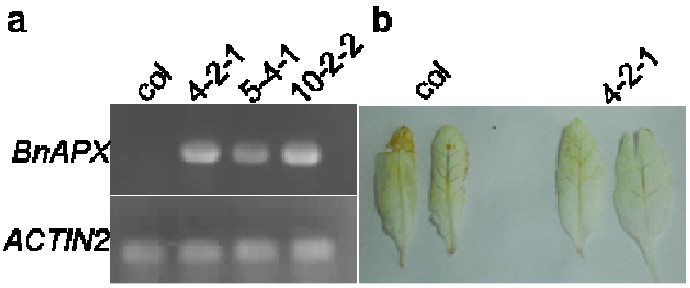

Figure 2. Overexpression of *BnAPX* decreased the H_2O_2 content. (a) The identification of transgenic *Arabidopsis* from transcriptional level by semi-quantitative PCR; col, wild type as the negative control; 4-2-1, 5-4-1 and 10-2-2, three homozygous transgenic lines; (b) staining of H_2O_2 with DAB under common conditions; col, the wild type; 4-2-1, the *BnAPX*-overexpressing line 4-2-1.

according to the instructions. Reverse transcription was performed by reverse transcriptase kit (MBI Fermentas, Canada) in accordance with the instructions. The transcription level of *BnAPX* was determined by semi-quantitative PCR analysis using the specific primers for *BnAPX* (DL-1:5′-AACAGAGGCTCTAGTAACACGG-3′, DL-2: 5′-ACTGGAACAAGTCGGCGAAA-3’). The β-ACTIN gene (ACT-1: 5’-TCTCGTTGTCCTCCTCACTT -3′, ACT-2: 5′-TATCATCAGCCTCAGCCATT -3′) was used as an internal control.

H_2O_2 staining with 3, 3’-diaminobenzidine tetrahydrochloride (DAB)

The protocol was modified from Guan and Scandalios (2000). Leaves or seedlings of *Arabidopsis* were excised and submerged into test tubes containing about 10 ml DAB solution. After about 12 h in the dark, they were washed with water and then transferred into clean test tubes with EtOH (95%) and the chlorophyll was extracted (Thordal-Christensen et al., 1997; Zhou et al., 2000).

Salt tolerance assay with transgenic plants

94 to 114 *Arabidopsis* seeds from wild type and T_2 homozygous transgenic *Arabidopsis* plants were plated on MS media supplemented with 0 or 100 mM NaCl after three days of jarovization at 4°C. Seeds were considered germinated when the radicles penetrated the seed coats and the germination rates were scored everyday under common condition. For root growth assay, wild type and T_2 homozygous transgenic seeds were germinated on MS solid medium. Four days after germination, the seedlings were transferred to MS solid medium supplemented with 0, 50, 100 and 150 mM NaCl respectively, for another 12 days. The tolerances of the plants were estimated mainly by measuring the root length and the H_2O_2 content. Three replicates were carried out and standard deviations were calculated. The data were analyzed and plotted in Microsoft Office software.

Drought tolerance assay with transgenic plants

After three days of jarovization at 4°C, seeds of both wild type and transgenic *Arabidopsis* were plated on MS media with or without 200 mM mannitol and the germination rate was scored. Three replicates were carried out and standard deviations were calculated. The data were analyzed and plotted in Microsoft Office software. To determine the drought tolerance of the seedlings, irrigation was stopped three days after germination in the soil. With the slow evaporation of the water, soil became water-deficient gradually, and the seedlings began to suffer the drought stress from the 2nd week. The water deficiency condition was kept and the H_2O_2 content was determined in the 3rd week, while the phenotype was observed in the 4th week.

RESULTS

Identification of transgenic *Arabidopsis*

After the *Agrobacterium*-mediated inflorescence dip, the T_0 seeds were obtained and plated on MS medium with 50 mg/L Kan. There were 21 individual Kan-resistant lines harvested, producing the true leaves and long roots (Figure 1b) and they were further checked by PCR. The upstream primer annealed to 35S promoter on pCAMBIA-2301-BnAPX and the downstream primer annealed to the *BnAPX* gene was used in the amplification. More also, a 550 bp band corresponding to the anticipated product in size was obtained from Kan-resistant plants, while nothing was gotten from the wild type (Figure 1c). This suggests that the *BnAPX* was inserted into the genome of *Arabidopsis* in sense orientation in the 3 Kan-resistant lines. The transgenic plants were transferred into soil. After selfing and screening of two successive generations (T_0 and T_1), the homozygous seeds were obtained, among which three lines named 4-2-1, 5-4-1 and 10-2-2 (T_2) appeared stronger, so they were selected for subsequent researches.

Transgenic plants suffered less H_2O_2 than wild type under normal conditions

As to the transcription level analysis, the semi-quantitative PCR was used. It showed that all of the three homozygous lines aforementioned had strong positive bands, while none band was observed in the wild type (Figure 2a), though the expressing levels were different

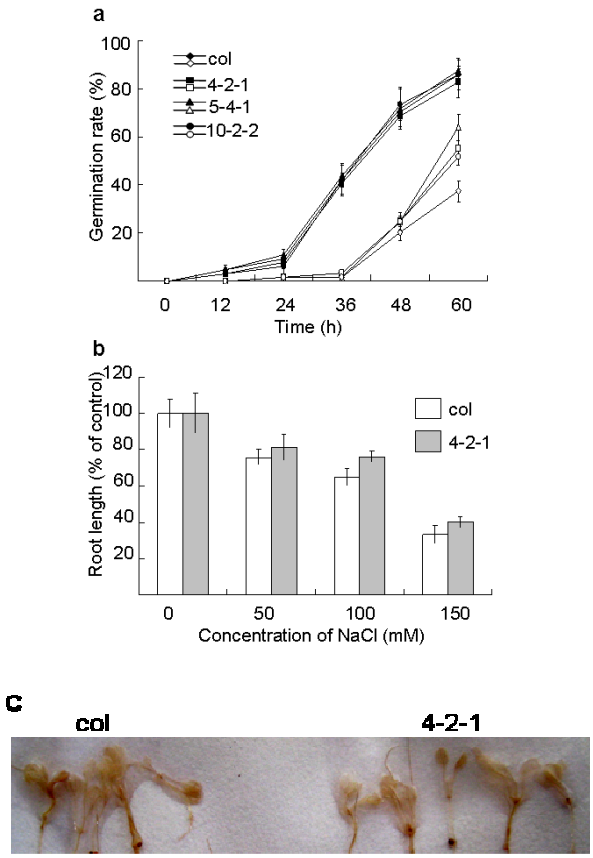

Figure 3. *BnAPX* enhanced salt tolerance in transgenic lines. (a) Germination analysis of wild type and transgenic *Arabidopsis* seeds under 100 mM NaCl stress; solid black symbols, seeds germinated on MS as control; hollow symbols, seeds germinated on MS+100 mM NaCl; (b) root lengths of the transgenic lines and the wild type under different NaCl concentrations; col, the wild type; 4-2-1, the *BnAPX*-overexpressing line 4-2-1; (c) staining of H_2O_2 with DAB after 100 mM NaCl stress; col, the wild type; 4-2-1, the *BnAPX*-overexpressing line 4-2-1. Results represent the mean of three replicates. Bars correspond to the standard deviation.

among the transgenic lines. This result was sufficient to demonstrate that *BnAPX* gene was constitutively and functionally expressed in the transgenic *Arabidopsis*.

Moreover, under normal conditions, the transgenic plants did not show any obvious differences from the wild type at the vegetative growth stage (data not shown). Since the main function of APX was scavenging the H_2O_2, it was therefore necessary to detect the endogenous H_2O_2 content in transgenic plants and the wild type. Here, DAB was involved in the staining to determine H_2O_2 content qualitatively. DAB reacted with H_2O_2 *in situ* and bronzing spots consequently appeared, so the H_2O_2 content could be determined according to the EtOH-bleached leaves (Guan and Scandalios, 2000; Zhang et al., 2009). As shown in Figure 2b, leaves from transgenic lines had lighter color than wild type under normal growth conditions; in other words, the transgenic lines suffered less H_2O_2. ROS was produced even in normal physiological and biochemical process (Asada and Takahashi, 1987). In the transgenic plants, the H_2O_2 was eliminated effectively, so the leaves had lighter color, which was due to the overexpression of *BnAPX*.

Overexpression of *BnAPX* enhanced germination rate of transgenic *Arabidopsis* seeds under salt stress

After three days of jarovization at 4°C, seeds of both transgenic plants and wild type were placed on MS medium of 0 and 100 mM NaCl, respectively. On the MS medium, the transgenic and wild type seeds germinated almost simultaneously, and both the germination rates were up to about 85% until 60 h (Figure 3a) but the germination rate of wild type was notably lower than that

of transgenic lines under 100 mM NaCl stress (Figure 3a). The germination rate of the wild type seeds was 37.5% until 60 h, while the lowest germination rate of the transgenic seeds was 51.6%, and those of the other two transgenic seeds were 55 and 64%, respectively. These results suggest that the seeds of wild type were more sensitive to salt stress than transgenic seeds, and the overexpression of *BnAPX* enhanced the tolerance of the transgenic *Arabidopsis* seeds to salt stress at the germinal stage.

Overexpression of *BnAPX* enhanced the salt tolerance of transgenic seedlings

As well known, plants suffer from growth inhibition under salt tress. In our research to determine the salt tolerance of *BnAPX*-overexpressing plants, the seeds of transgenic lines and the wild type were first plated on MS medium, a procedure that ensured that the seeds of both lines germinated and grew in spite of the step. Four days after germination, they were transferred to MS medium with 0, 50, 100 and 150 mM NaCl, respectively. It was found that no matter the transgenic lines or the wild type, the root lengths suffered with varying degrees of inhibition and the higher the NaCl concentration, the shorter root length the seedlings had. However, it was observed that under any NaCl concentration, the transgenic lines had longer roots than the wild type (Figure 3b). This suggests that under the protection of *BnAPX*, transgenic *Arabidopsis* relieved the inhibition phenomenon induced by NaCl stress. DAB staining showed transgenic seedlings suffered less H_2O_2 than the wild type (Figure 3c) after NaCl treatment.

Overexpression of *BnAPX* enhanced drought tolerance of *Arabidopsis*

Similar to the situation under NaCl stress, the germination of wild type under 200 mM mannitol stress was significantly slower than that of transgenic lines (Figure 4a). The germination rate of the wild type seeds was 35.9%, while the germination rates of the three transgenic lines were 58, 68 and 65%, respectively, which were much higher than that of the wild type. As for the seedlings, the withered phenomenon appeared among the two week-old wild type lines under drought stress for another two weeks, and the leaves became tawny; while the transgenic lines showed much healthier properties (Figure 4b). DAB staining showed that the level of H_2O_2 was lower in transgenic seedlings than in the wild type (Figure 4c) under drought stress. These results suggest that the seeds and seedlings of wild type were more sensitive to drought stress than transgenic seeds, and the overexpression of *BnAPX* enhanced the tolerance of the transgenic *Arabidopsis* to drought stress.

DISCUSSION

Researches on APX started from 1976 (Foyer and Hailiwell, 1976), and it has received greater attention because of its important physiological functions. Studies on APX from many kinds of plants were reported, but studies on APX from *Brassica napus*, the most important oil crop in China, seemed rare. In previous researches in our laboratory, Liu et al. (2010, 2011) cloned the *BnAPX* and found that the amino acid sequence of *BnAPX* shared 77% identity with thylakoidal APX from *Arabidopsis*, and it was localized in chloroplasts. Hence *BnAPX* was considered *tAPX* isozyme in *B. napus*. Moreover, to further study its functions in higher plant, in this research *BnAPX* was introduced into model plant *Arabidopsis* (Figure 1c) and overexpressed (Figure 2a). It was found that under normal conditions, there was no obvious physiological difference between transgenic plants and wild type in the vegetative growth stage (data not shown). This result was consistent with the findings of *tAPX*-overexpressing lines (Murgia et al., 2004). But since APX is a key enzyme scavenging H_2O_2 especially in chloroplasts, it was necessary to detect the H_2O_2 content *in vivo*. The DAB staining showed that bleached leaves from transgenic lines had lighter color than wild type (Figure 2b), demonstrating that the overexpression of *BnAPX* in the transgenic lines enhanced the capability of scavenging endogenous H_2O_2, though phenotype showed no difference.

H_2O_2 is a non-polar molecule, and it can travel across biological membranes, distribute in different cell compartments (Henzler and Steudle, 2000) and can be accumulated because of the relatively stable chemical property. Its accumulation can interfere with various kinds of normal physiological processes and injure the plants (Pnueli et al., 2003; Sun et al., 2010). Xu et al. (2008) found that *Arabidopsis* carrying a peroxisomal APX (*HvAPX1*) from barley was more salt-tolerant than the wild type. As for the mechanisms of salt tolerance, they thought it was not because of the maintenance and reestablishment of cellular ion homeostasis but the reduction of oxidative stress injury. In other words, scavenging ROS can enhance salt tolerance in plants. Since it was known that *BnAPX* scavenged more H_2O_2 in transgenic *Arabidopsis*, it was consequent to determine whether *BnAPX* enhanced the salt tolerance. To this end, we subjected the four-day-old seedlings to 0, 50, 100, 150 mM NaCl, respectively. Plants suffered growth inhibition under salt stress, and here the root length was used as the main feature to present it. After 12 days, it was found that both transgenic seedlings and the wild type seedlings shortened the root under every concentration of NaCl stress, but transgenic seedlings had longer roots than the wild type under any concentration of NaCl (Figure 3b). On the one hand, it demonstrated that *BnAPX* enhanced the salt tolerance of transgenic lines to certain degree; although the protection

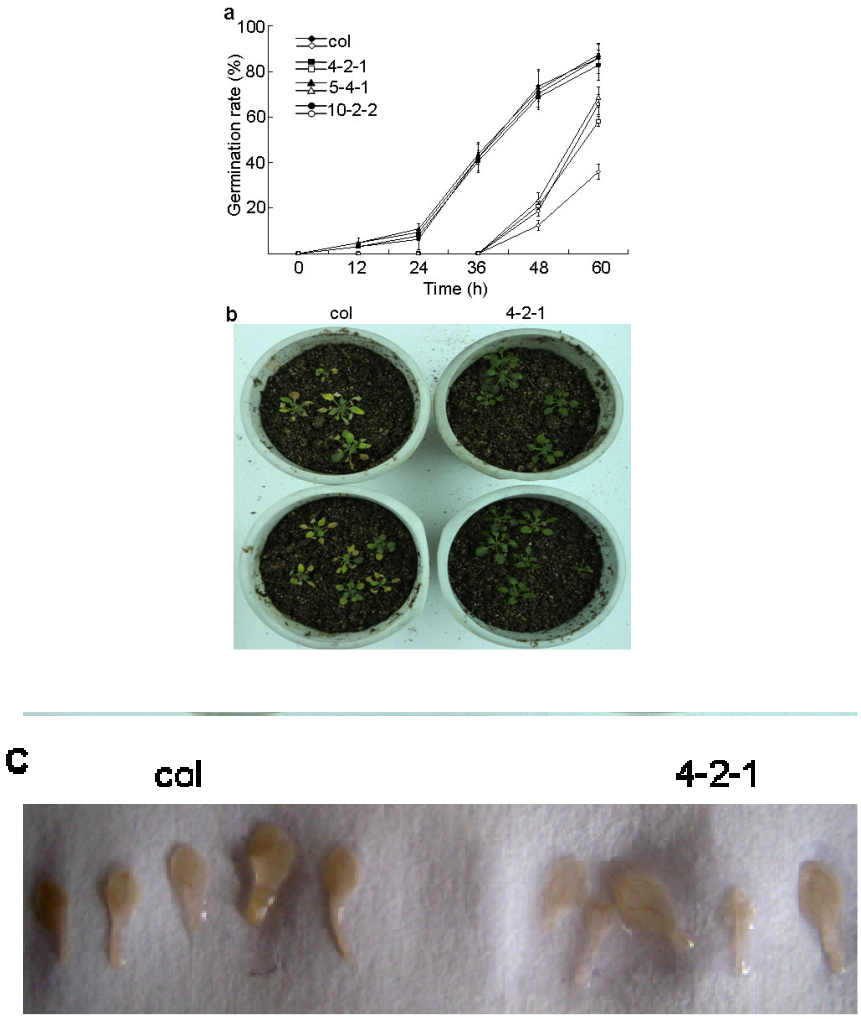

Figure 4. *BnAPX* enhanced drought tolerance in transgenic lines. (a) Germination analysis of wild type and transgenic *Arabidopsis* seeds under mannitol simulated drought stress; solid black symbols, seeds germinated on MS as control; hollow symbols, seeds germinated on MS+200 mM mannitol; (b) two-week-old seedlings under drought stress for another two weeks; col, the wild type; 4-2-1, the *BnAPX*-overexpressing line 4-2-1; (c) staining of H_2O_2 with DAB under drought stress; col, the wild type; 4-2-1, the *BnAPX*-overexpressing line 4-2-1. Results represent the mean of three replicates. Bars correspond to the standard deviation.

ability was limited. As to the drought tolerance, the difference was more obvious. It can be phenotypically identified that the transgenic seedlings kept greener and healthier than the wild type (Figure 4b). According to the opinion of Xu et al. (2008), we ascribed the tolerances to the enhanced capability of eliminating H_2O_2 (Figure 3c and 4c). Another impact of environmental stress on plants was to reduce the seed germination rate. Here the transgenic seeds presented higher germination rate under either 100 mM NaCl simulating salt stress (Figure 3a) or 200 mM mannitol simulating drought stress (Figure 4a). To sum up, *BnAPX* enhanced the salt and drought stresses in transgenic *Arabidopsis*, in both germination stage and vegetative growth stage of seedlings, and the enhanced tolerances were due to the increasing ability of H_2O_2 elimination under environmental stresses (Figure 3c, 4c).

Salinity is one of the most crucial abiotic stresses in agriculture, and there are about 20% of the world's cultivated lands and nearly 50% of all irrigated lands are affected by it (Zhu, 2000, 2001). Salt stress usually interrupts the endocellular ionic equilibrium and osmotic equilibrium, inhibits the growth and metabolism of plants, or secondarily is involves in consequent oxidative stress, or even death (Niu et al., 1995; Glenn et al., 1999; Zhu, 2001) among which the inhibition of growth is most significant. This research, however, provides a probability that *B. napus* could survive and grow normally under

environmental stress so that the yield is assured. Some other efforts are underway and the researchers have already made useful discoveries. For example, Wani and Gosal (2011) found that the commercial rice cultivar PAU 201 with introduced *OsglyII* gene showed tolerance to salt stress both at calli and plantlet level and they also optimized some protocols before the selection process for both salt and drought tolerances (Wani et al., 2010a, b). In any case, compared with irrigation or improving soil drainage, genetically modifying the crop seems more promising (Zhu, 2000; Gao et al., 2003).

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