DOI: 10.1007/s10535-015-0489-y BIOLOGIA PLANTARUM 59 (2): 305-315, 2015

Overexpression of the genes coding ascorbate peroxidase from *Brassica campestris* enhances heat tolerance in transgenic *Arabidopsis thaliana*

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Abstract

Previously, the ascorbate peroxidase (APX1) activity and gene expression in Chinese cabbage (*Brassica campestris*, *Bc*) heat-tolerant cv. ASVEG2 were found to be significantly higher than in heat-sensitive cv. RN720 under a heat stress. Furthermore, *BcAPX*2 and *BcAPX*3, isoforms of *BcAPX*1**,** were cloned in this study. Our objective was to transfer *BcAPX* cDNA under the control of the ubiquitin promoter to *Arabidopsis via Agrobacterium tumefaciens* strain GV3101. We found that *BcAPX* genes were overexpressed in transgenic *Arabidopsi*s, and the expression of *APX*, and the APX activity in transgenic lines were higher than in non-transgenic (NT) plants under high temperatures. The chlorophyll content and the germination rate were significantly higher, and the malondialdehyde content was lower in *BcAPX*1-3, 2-1, and 3-5 lines subjected to the heat-stress treatment than those in the NT plants. Compared to the NT plants, a lower heat-induced H_2O_2 accumulation was detected by diaminobenzidine staining in leaves of the transgenic lines with a high APX activity indicating that the overexpression of *BcAPX* in *Arabidopsis* could enhance heat tolerance by eliminating H_2O_2.

Additional key words: *Agrobacterium*-mediated transformation, Chinese cabbage, chlorophyll, hydrogen peroxide, malondialdehyde.

Introduction

Environmental stresses are limiting for horticultural productivity worldwide. Among them high temperatures cause morphological, physiological, and biochemical changes including leaf aging, membrane damage, chlorophyll degradation, decreased photosynthetic efficiency (Djanaguiraman *et al.* 2009), as well as the accumulation of reactive oxygen species (ROS; Volkov *et al.* 2006, Kreslavski *et al.* 2009, Suzuki *et al.* 2012). The excess production of ROS, such as superoxide radicals, hydrogen peroxide, singlet oxygen, and hydroxyl radicals, causes oxidative damage to cellular components (Shigeoka *et al.* 2002), however, they are necessary for signaling, *e.g.*, H_2O_2 triggers programmed cell death in plants (Wu *et al.* 2012, Ishikawa *et al.* 2013). Many physiological changes occurring during heat stress results in increased heat tolerance. Heat tolerance can be achieved by several adaptive mechanisms, and the modulation of antioxidant system could be part of them. One of these might be an increased expression of ascorbate peroxidase (*APX*) genes to protect against oxidative stress. APX (EC 1.11.1.11) catalyzes the reduction of H_2O_2 using ascorbate as electron donor to yield water and oxidized ascorbate. The ascorbate-glutathione cycle has been shown to be of a great importance in multiple stress reactions (Blokhina *et al.* 2003).

Recently, successful attempts were made to transfer genes involved in heat-stress tolerance into plants, and the resulting transgenic plants were reported to exhibit enhanced tolerance to heat and related stresses (Zhao *et al.*2009, Iseri *et al.* 2011, Yildiztugay *et al.* 2011). The overexpression of tomato GDP-mannose pyrophospho-

Submitted 22 March 2014, *last revision* 6 September 2014, *accepted* 9 September 2014.
Abbreviations: APX - ascorbate peroxidase; CAT - catalase; DAB - diaminobenzidine; MDA - malondialdehyde; NT - non-transgenic; ROS - reactive oxygen species; SOD - superoxide dismutase.
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rylase (GMPase) in tobacco increased the activities of superoxide dismutase (SOD) and APX, and conferred transgenic plant resistance to high temperature injury (Wang *et al.* 2011). The overexpressions of *SOD* and *APX* in transgenic tall fescue plants enhanced antioxidant enzyme activities and conferred better tolerance to oxidative stress (Lee *et al.* 2007a). Induction of glutathione (GSH) produced by rice γ-glutamylcysteine synthetase (*OsECS*) overexpression leads to improved redox homeostasis through detoxification of ROS produced by high salinity (Choe *et al.* 2013). In addition, transgenic Chinese cabbage plants that overexpress genes coding SOD and CAT have been found to be more tolerant to salt stress (Tseng *et al.* 2007). Transgenic potato plants expressing both *SOD* and *APX* genes in chloroplasts under the control of an oxidative stress-inducible peroxidase promoter show enhanced tolerance to methyl viologen-induced oxidative stress and high temperature (Kim *et al.* 2010). The seed-specific overexpressions of *SOD* and *CAT* genes in *Arabidopsis* enhance oxidation stress tolerance during germination and early seedling growth (Xi *et al.* 2010). The over-expression of *Arabidopsis* glutaredoxin *GRXS*17 in tomato increases CAT activity and effectively reduces the accumulation of H_2O_2, so transgenic plants show significant heat tolerance (Wu *et al.* 2012). The transformation of alfalfa by rice *OsAPX*2 enhances its tolerance to salinity (Guan *et al.* 2012). The overexpression of rice *OsAPXa* gene reduces the accumulation of H_2O_2 and increases chilling tolerance (Sato *et al.* 2011).

Previously, we studied the heat response in Chinese cabbage cv. ASVEG2 (heat-tolerant) and cv. RN720 (heat-sensitive) to compare the antioxidant activity and physiological mechanisms under a heat stress (Lin *et al.* 2010). Results demonstrated that RNA levels and the APX activity of ASVEG2 plants treated at 35 and 40 °C for 24 to 72 h are significantly higher than those of RN720 plants. The experiments followed by cloning the full length of *BcAPX* (GenBank accession No. AB901369). The aim of the current study was to further investigate the heat tolerance induced by the *APX* gene by transferring *BcAPX* cDNA from ASVEG2 plants into *Arabidopsis via* an *Agrobacterium*-mediated transformation. A ubiquitin promoter was used to drive *BcAPX* in transgenic *Arabidopsis* plants. The hypothesis was that the overexpression of *BcAPX* in the transgenic plants would significantly increase heat tolerance compared to non-transgenic plants. The characterization and functional analysis of *APX* genes should facilitate our understanding of heat-response mechanisms in plants.

Materials and methods

Plants, culturing, and heat-stress treatment: Seeds of *Arabidopsis thaliana* L. ecotype Columbia and Chinese cabbage (*Brassica campestris* L. ssp. *pekinensis* cvs. ASVEG2 and RN720) from the Asian Vegetable Research and Development Center, Tainan, Taiwan, were used. Culture practices including watering and fertilization were described in our previous paper (Lin *et al.* 2010). Briefly, seeds were sterilized with 1.5 % (m/v) sodium hypochlorite, rinsed with distilled-deionized H_2O, sown in a commercial potting soil mixture, and grown in a growth chamber under an irradiance of 300 μmol m^{-2} s^{-1}, a 14-h photoperiod, day/night temperatures of 25/20 °C, and a relative humidity of 80 %. The plants were watered three times a week and fertilized (N:P:K, 17:36:39) once a week to maintain optimal growth for 21 d. To induce a heat stress, the plants were exposed to 40 °C for 5 h (Chiang *et al.* 2014).

Amplification and cloning the *BcAPX* genes: Total RNA was isolated from 0.1 g of ASVEG2 leaves with a *Qiagen* (Valencia, CA, USA) RNeasy plant mini kit and poly $(A)^+$ mRNA was then extracted from total RNA with a *Qiagen Oligotex* mini kit according to the vendor instructions. First-strand cDNA was synthesized from 1 μg of total RNA using retroscript-reverse transcription for the reverse-transcription polymerase chain reaction (RT-PCR) with an oligo (dT) primer according to the manufacturer instructions (*Ambion*, Austin, TX, USA). Paired degenerated primers (*BcAPX*-5F and *BcAPX*-3R, Table 1) were used for amplification. The amplification of the APX gene was based on a previous study (Lin *et al.* 2010). Briefly, PCR was carried out in an *Eppendorf* master-cycler gradient thermal cycler (Hamburg, Germany) with the following thermal program: an initial denaturation at 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 40 s, with a final extension at 72 °C for 10 min. The products were electrophoretically separated on 1.5 % (m/v) agarose gels, and the predicted size of 753 bp of the *APX* gene was verified with a 100-bp DNA ladder marker. The RT-PCR products were then cloned into the pGEM-T vector (*Promega,* Madison, WI, USA), followed by transfer into competent DH5α cells. Blue and white screening was then used to identify the transformant colonies. After the plasmid DNA extraction, the PCR amplification of the transformed colonies was carried out with the *BcAPX* gene being confirmed by DNA sequencing (*Tri-I Biotech*, Taipei, Taiwan). A previously cloned gene under a heat stress was called *BcAPX1* (Lin *et al.* 2010), and the cloned APX genes from this study were named *BcAPX2* and *BcAPX3* (GenBank accession Nos. AB901370 and AB901371, respectively).

Plasmid construction, pENRT TOPO cloning, and LR recombination reaction: An *Escherichia coli* strain *TOP 10* (*Invitrogen*, Carlsbad, CA, USA) were used for gene construction and transformation. The destination vector was pAHC18 containing *Luc* cDNA which was fused between the *Ubi* promoter and the nopalin synthase gene (*Nos*) terminator. A hemagglutinin (HA) tag containing

nine amino acids (YPYDVPDYA) was directed using the oligonucleotides HA5F (5'-TACCCATACGACGTT CCAGACTACGCT-3') as the 5' primer and HA3R (5'-AGCGTAGTCTGGAACGTCGTATGGGTA-3') as the 3' primer to form a primer dimer, followed by ligation to *ccd* gene (*Invitrogen*) to enable high-efficient cloning. The construct was then introduced into pAHC18 (digested with *Bam*HI to remove the *Luc* cDNA insert and blunt-end), and pAHC18/HA-ccdB was generated. The cauliflower mosaic virus 35S RNA (CaMV35S) promoter, hygromycin B phosphotransferase (*hpt)* coding sequence, and tumor morphology large 3'UTR (*tml*) fusion gene were excised with *Eco*RI from pTRA151 (Zheng *et al*. 1991), subcloned into the *Eco*RI site of the binary vector pPZP200 (Hajdukiewicz *et al*. 1994), and used to generate pPZP200/hpt. The pAHC18/HA-ccdB vector was then cut by *Hind*III, followed by ligation to pPZP200/hpt (previously cut by *Hind*III) to generate a binary vector, and pPZP200/Ubi-HA-*ccdB*-Nos-35S-*hpt*-tml. *BcAPX*s (1 - 3) were constructed for plant transformation by the *Invitrogen TOPO® Gateway* expression vector system. This system was separated into two parts for *pENTR® TOPO* cloning and an LR exchange reaction to the destination vector. *BcAPX*s were amplified with the specific paired primers (*BcAPX*-5F and *BcAPX*-3R, Table 1) from the above-described RT-PCR using high-fidelity DNA polymerase (*New England Biolabs*, Beverly, MA, USA). *BcAPX* fragments (pGEM-*BcAPX*) were amplified for 753 bp in which water was used as negative control. PCR products were purified using a *SNAP* gel purification kit (*Invitrogen*), ligated to the *pENTR TOPO* vector, and transformed into *TOP10 E. coli*-competent cells. After kanamycin screening, plasmid DNA from the transformed *E. coli* was extracted using a plasmid *Miniprep* kit (*GeneMark*, Atlanta, GA, USA). The APX fragment was amplified using paired primers (*BcAPX*-5F and *BcAPX*-3R, Table 1) inserted into pENTR plasmids. APX cDNA sequencing pENTR-*BcAPX* was then checked. Constructed plasmids were named as clones, pENTR-*BcAPX*s (Fig. 1*A* Suppl.). After pENTR cloning, an LR recombination reaction was performed with a *Gateway® LR Clonase™* enzyme mix kit (*Invitrogen*). The pENTR-*BcAPX* plasmids were exchanged and cloned into the *ccdB* region of the destination vector, pPZP200/Ubi-HA-*ccdb*-Nos-35S-*hpt*-tml, by LR site-specific recombi-nation. After spetinomycin screening the colony, the plasmid insertion was confirmed by PCR and DNA sequences of both pPZP200/Ubi-HA-*APXs*-Nos-35S-*hpt*-tml (pPZP200-*BcAPX*s) clones (Fig. 1*B* Suppl.).

***Agrobacterium*-mediated transformation and genomic PCR analysis:** A freeze-thaw method was used to transform plasmids into *Agrobacterium tumefaciens* L. strain GV3101, and transformation-competent *Agrobacterium* cells were prepared according to Jyothishwaran *et al.* (2007). The PCR amplification of *BcAPX* was performed following plasmid extraction. The *Agrobacterium*-containing pPZP200-*BcAPX* plasmids were delivered into *Arabidopsis* by floral dipping protocols based on the Clough and Bent (1998) method. T1 seeds were collected and heated until dry. Transgenic seedlings were selected on a MS medium with 50 mg dm^{-3} hygromycin and the efficiency of transformation was calculated. Leaves of T1 transgenic *Arabidopsis* plants were ground to a fine powder with a mortar and pestle in liquid nitrogen. Genomic DNA was prepared essentially as previously described (Chua *et al.* 1990). A PCR analysis was performed using specific primers *BcAPX*5F/*BcAPX*3R (Table 1) to amplify the *APX* gene. The N-terminal of *BcAPX* was fused with an HA tag (Fig. 1*B* Suppl.). The amplification of the endogenous *Arabidopsis APX* was avoided by using HA-*BcAPX* 5F and *BcAPX* 3R for PCR. PCR was carried out in an *Eppendorf Mastercycler* gradient thermal cycler with the following thermal program: an initial denaturation at 94 °C for 3 min followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s, with a final extension at 72 °C for 10 min. PCR products were electrophoretically separated on a 1.5 % agarose gel, and the predicted size of 801 bp (*BcAPX*) was verified with a 100-bp DNA ladder of a DNA marker.

Real-time quantitative PCR and quantification of RNA: A real-time qPCR analysis was employed to quantify and validate relative changes in the expression of the *APX* gene with heat induction. Total RNA was extracted from 3-week-old leaves of both transgenic and non-transgenic (control) *Arabidopsis* plants after 40 °C for 5 h. First-strand cDNA was synthesized using the *Ambion* kit mentioned earlier. A real-time qPCR was carried out according to Lin *et al.* (2010). The above-mentioned primers, HA-*BcAPX*5F/*BcAPX*3R (Table 1), were used for the real-time qPCR. Briefly, each reaction (20 mm^3) contained 1 mm^3 of cDNA, 9 mm^3 of distilled deionized H_2O, 2 mm^3 of *Primer Mix* (10×), 4 mm^3 of 5 × *SYBR Green I*, 1.6 mm^3 of $MgCl_2$ (3 mM), 0.4 mm^3 of the enzyme mix, and 2 mm^3 of resolution solution from a *Roche* (Basel, Switzerland) RNA amplification kit. The amplification program consisted of 1 cycle of 94 °C for 1 min for pre-incubation followed by 40 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 60 s. Six replicates were performed for each cDNA sample, and template-free and negative controls were used. To normalize the total amount of cDNA in each reaction, the *actin-8* gene from *Arabidopsis* (*AtActin8*, Table 1) was co-amplified as internal control. These relative RNA quantities of RNA samples are presented as "expression" values, allowing comparisons of relative RNA amounts among transgenic *APX* lines and non-transgenic (NT) plants. Threshold cycles (CTs) were determined using the *7000 SDS* system software, and $2^{-\Delta\Delta CT}$ values were calculated (Livak and Schmittgen 2001).

Western blot analysis: The protein extraction protocol was modified from Chen *et al.* (2006). Total soluble proteins were prepared from 300 mg of fresh leaves of both transgenic and non-transgenic (NT) plants subjected

to 40 °C for 5 h, and quantitated with a Bradford protein assay kit (*BioRad*, Hercules, CA, USA). Total proteins (40 μg) in each well were electrophoresed on a 4 % (m/v) stacking gel and 12.5 % (m/v) resolving gel (sodium dodecylsulfate polyacrylamide gel electrophoresis; SDS-PAGE) on a *Mini PROTEAN III* equipment (*Bio-Rad*). Following electrophoresis, proteins were transferred to an *Immobilon-P* transfer membrane (*Millipore*, Billerica, MA, USA). For APX immunodetection, the membrane was incubated at 4 °C overnight with a mouse anti-HA antibody (*Sigma*, St. Louis, USA). Bands were detected with an anti-mouse immunoglobulin G (IgG) peroxidase-conjugated secondary antibody for 1 h. The 3,3',5,5' tetramethyl benzidine (TMB) substrate from a *TMB* kit (*BioLegend*, San Diego, CA, USA) was used for staining, as it is catalyzed by peroxidase to produce a pale-blue color.

APX activity assay: APX activity was assessed by measuring the initial rate of disappearance of ascorbate following protocols from Asada (1984). Leaf samples (40 mg) from 3-week-old transgenic and NT plants subjected to 40 °C for 5 h were extracted with 4 cm^3 of a sodium phosphate buffer (50 mM, pH 6.8) to extract enzymes. The APX assay reaction mixture (3 cm^3) contained 1 cm^3 of a 150 mM potassium phosphate buffer (pH 7.0), 1 cm^3 of 1.5 mM ascorbate, 0.9 cm^3 of distilled deionized H_2O, and 0.1 cm^3 of the enzyme extract. A decrease in ascorbate was reflected as a decline in absorbance at 290 nm, and the activity was calculated with the coefficient of absorbance of 2.8 mM^{-1} cm^{-1}. One unit of APX activity was ΔA_{290} per min.

Diaminobenzidine (DAB) staining assay for H_2O_2 accumulation: DAB staining procedure were modified from Thordal-Christensen *et al.* (1997). Leaves from transgenic and NT *Arabidopsis* (3-week-old) plants were heated to 40 °C for 5 h, and a DAB staining solution was added. After incubation overnight, the formation of local brown spots in the leaves by H_2O_2 was visualized. Quantitative analyses of leaves stained with DAB were made by scanning the leaves with a computing laser densitometer using the *Image Quant v. 3.19.4* software (*Molecular Dynamics*, Sunnyvale, CA). Each experiment was performed with three replicates.

Determination of chlorophyll content, lipid peroxide, and germination rate: All plants were grown at 25/22 °C for 21 d, then a heat stress was applied for 3 d (40 °C for 5 h each day), followed by recovery for 2 h. Chlorophyll was eluted from NT and transgenic leaves with 5 cm^3 of 80 % acetone at 4 °C overnight and then centrifuged at 13 000 *g* for 5 min. The supernatants were measured at 663 and 645 nm with a spectrophotometer (*U-2000*, *Hitachi*, Tokyo, Japan) according to Porra *et al.* (1989).

Malondialdehyde (MDA) is the final decomposition product of lipid peroxidation and is used as an index for the status of lipid peroxidation. Thiobarbituric acid (TBA)-reactive substances representing lipid peroxidation products were extracted by homogenization of 0.2 g of leaf in 5 cm^3 of 0.6 % (m/v) TBA solution in 10 % (m/v) trichloroacetic acid (TCA). The mixture was heated to 95 °C for 30 min and the reaction stopped in an ice bath. The cooled mixture was centrifuged at 13 000 *g* at 25 °C for 10 min, and the absorbances of the supernatant were read at 532 and 600 nm. After subtracting the value at 600 nm, the MDA content was determined using the coefficient of absorbance of 155 mM^{-1} cm^{-1} (Kosugi and Kikugawa 1985).

Both transgenic (T1) and NT *Arabidopsis* seeds were sterilized with 1.5 % (m/v) sodium hypochlorite and rinsed with distilled deionized H_2O. One hundred seeds were placed on a Petri dish containing an MS medium and incubated at 40 °C for 5 h. The seeds were germinated at 22 °C under a 16-h photoperiod for a week and the germination rate was calculated.

Statistical analysis: Three replications of three-week-old plants of each transgenic (T1) and NT line were investigated to obtain the RNA content, APX activity, chlorophyll and MDA content, and germinating rate. Data are presented as means of at least two independent sets of experiments with similar results. Statistical significance was calculated by the paired two-tailed Student's *t*-test for RNA content and APX activity. A value of $P \leq 0.05$ indicated statistical significance. Measurements of the chlorophyll and MDA content and germination rates were analyzed by the analysis of variance (*ANOVA*) and means were separated by the least significant difference (LSD) test at $\alpha = 0.05$ using *PC SAS v. 8.2* (*SAS Institute*, Cary, NC, USA).

Table 1. Paired primers used in the study (F - forward, R - reverse).

Name	Primer	Sequence	Expected size [bp]
pENTTR/BcAPX and pPZP200/BcAPX	*BcAPX* 5F	5'-CACCATGGATCCATACAAGT-3'	753
	BcAPX 3R	5'-TTAGATGCTTGGTCTCACGT-3'	
pENTTR/BcAPX and pPZP200/BcAPX	HA*BcAPX* 5F	5'-CATACGATGTTCCAGTACC-3'	801
	HA*BcAPX* 3R	5'-TTAGATGCTTGGTCTCACGT-3'	
AtActin8	Actin8 5F	5'-CCCAAAAGCCAACAGAGAGA-3'	140
	Actin8 3R	5'-CATCACCAGAGTCCAACACAAT-3'	

Results

Previously, the *BcAPX*1 gene from heat-tolerant cv. ASVEG2 was cloned and its up-regulation in heat-sensitive cv. RN720 has been found in response to a heat-stress (Lin *et al.* 2010). In the present study, the full-length cDNA of *BcAPX*2 and *BcAPX*3, isoforms to *BcAPX*1, were cloned from ASVEG2 under the heat-stress treatment. Fig. 2*A* Suppl. illustrates that there was only a 2-bp difference in nucleotide sequences in an open-reading frame from *BcAPX*1, and a two amino acid difference when nucleotides were translated into amino acids (Fig. 2*B* Suppl.). After the transformation of *Arabidopsis* by floral dipping, seeds were collected and germinated on an MS medium containing 50 mg dm^{-3} hygromycin for 14 d. The non-transgenic (NT) seedlings were pale and non-viable (data not shown), but shoots of the transgenic seedlings were green and appeared healthy. The seedlings were transplanted into soil for continued growth. Transformation efficiencies of hygromycin-resistant transgenic lines in *BcAPX*1, *BcAPX*2, and *BcAPX*3 were 1, 0.6, and 0.6 %, respectively (Table 2). Eleven healthy, robust, independent transgenic lines of *Arabidopsis* were generated and designated *BcAPX*1-1, 1-3, 1-4, 1-5, 1-8, 2-1, 2-2, 2-3, 3-2, 3-3, and 3-5. These T1 transgenic lines were detected by genomic DNA PCR amplification and electrophoresis. All of the transgenic *Arabidopsis* lines displayed the expected size (801 bp) of the HA-*BcAPX* gene (Fig. 3 Suppl.). The transcription of *APX* was analyzed by real-time qPCR to verify whether the gene was differentially expressed in ASVEG2 plants under the 40 °C exposure for 5 h. Data were normalized with respect to the RNA content of *actin*, a housekeeping gene that is consistently expressed in plants. The RNA content of heat-induced *APX* was 3.95-fold higher than in the non-heated plants indicating that the expression of *APX* was significantly upregulated by the high temperature (Fig. 1*A*).

To investigate whether the *BcAPX* genes from ASVEG2 were expressed in transformed *Arabidopsis*, a real-time qPCR analysis was performed with extracted RNA from 21-d-old NT and eleven transgenic lines receiving 40 °C exposure for 5 h. The *BcAPX* genes were constitutively expressed in the transgenic *Arabidopsis*, and the RNA levels of *BcAPX*1-1 (2.77), 1-3 (1.86), 1-4 (1.55), 1-5 (3.42), 1-8 (3.50), 2-1 (4.06), 2-3 (1.38), 3-2 (3.80), 3-3 (1.48), and 3-5 (3.18) lines were significantly higher than in the non-transgenic plants and in the

Table 2. Efficiency of plasmid transformation in *Arabidopsis*

Plasmids	Total number of infected explants	Hygromycin resistant plants	Transformation efficiency [%]
pPZP200-BcAPX1	500	5.0	1.0
pPZP200-BcAPX2	500	0.6	0.6
pPZP200-BcAPX3	500	3.0	0.6

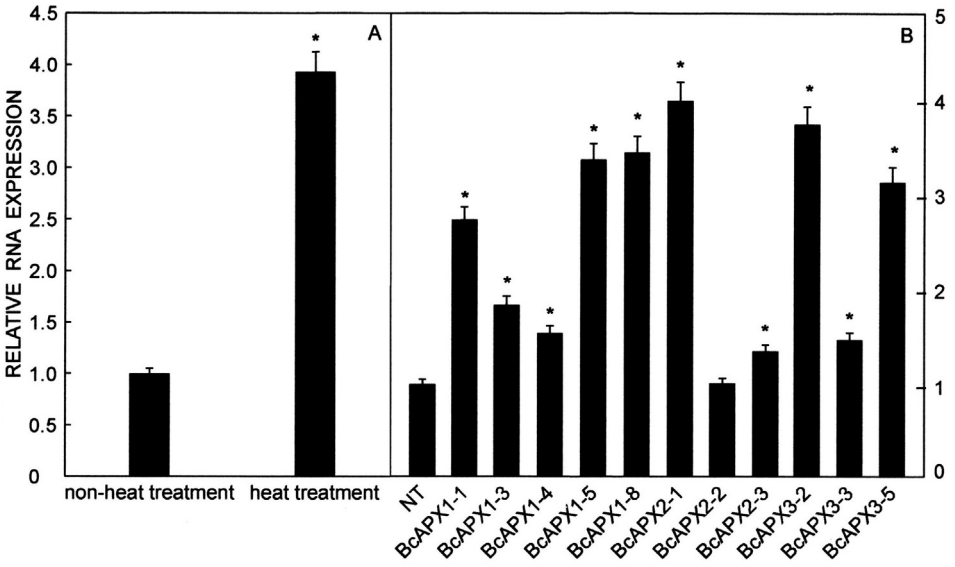

Fig. 1. The expression of *APX* in ASVEG2 plants under non-heat and heat-stress treatments (*A*), and in non-transgenic (NT) *Arabidopsis* plants and transgenic *Arabidopsis* lines under a heat-stress treatment (*B*). Total RNA in all the tested plants was extracted from 21-d-old plants subjected to 40 °C for 5 h. The amplification of endogenous *Arabidopsis* APX was avoided by using HA-*BcAPX* 5F and *BcAPX* 3R for PCR. The *actin-8* gene was used as internal control. Means ± SD, $n = 3$, * - significant differences ($P \leq 0.05$) according to the Student's *t*-test.

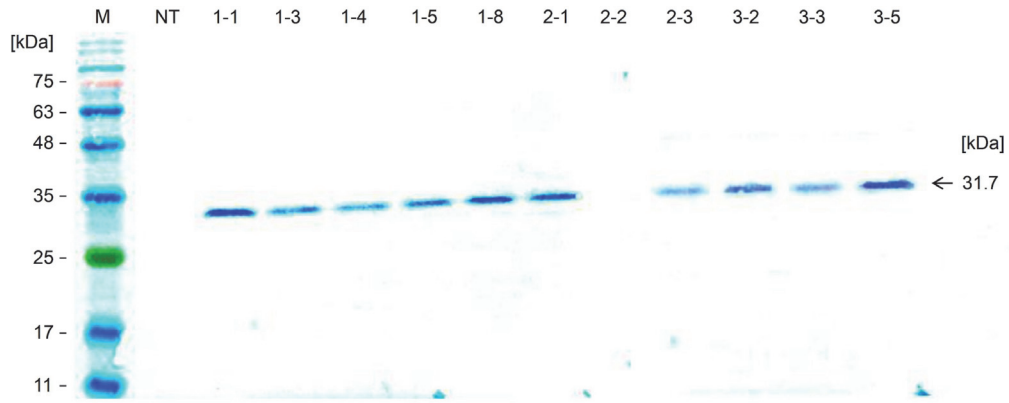

Fig. 2. The Western blot analysis of *BcAPX* transgenic lines (1-1, 1-3, 1-4, 1-5, 1-8, 2-1, 2-2, 2-3, 3-2, 3-3, and 3-5) compared to non-transgenic (NT) *Arabidopsis* plants (40 μg of total proteins were used in each sample). The molecular mass of *BcAPX* at 31.7 kDa is indicated by the *arrowhead*.

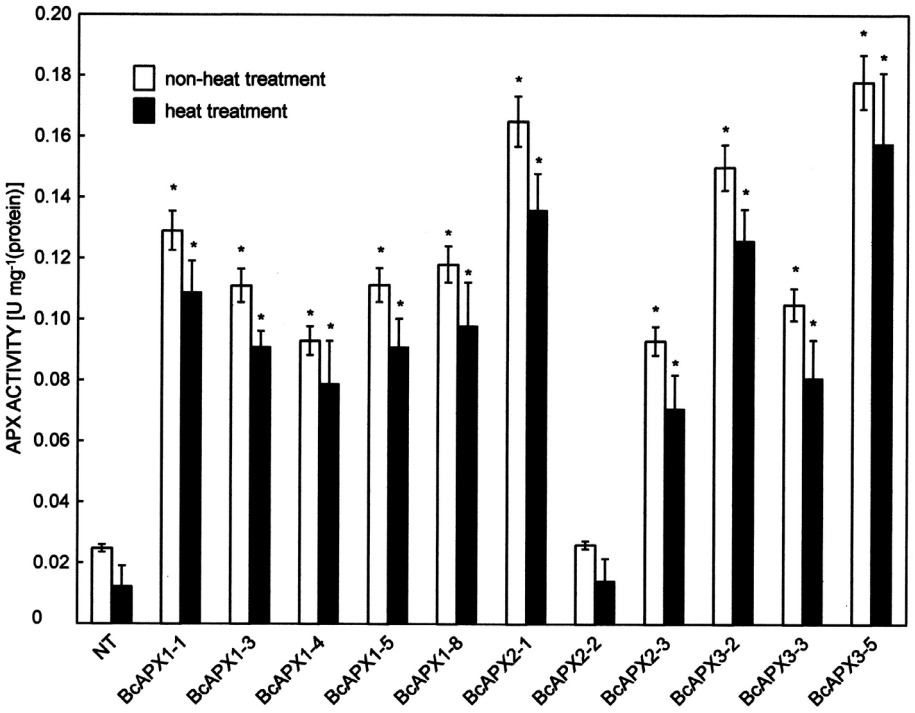

Fig. 3. The APX activity in leaves of NT and BcAPX transgenic lines after their exposure to 40 °C for 5 h (a heat treatment) in comparison with non-heated plants. Means ± SD, $n = 3$, * - significant difference at $P \leq 0.05$ according to the Student's *t*-test.

*BcAPX*2-2 line under the heat stress (Fig. 1*B*). To estimate the relative amounts of the BcAPX protein accumulating in the transgenic *Arabidopsis* lines, a Western blot analysis was conducted by using antiserum anti-HA to identify the *BcAPX* expression (Fig. 2). A clear band (31.7 kDa) appeared in all transgenic lines (*BcAPX*1-1, 1-3, 1-4, 1-5, 1-8, 2-1, 2-3, 3-2, 3-3, and 3-5), but this protein was not detected in the NT and *BcAPX*2-2 plants. In addition, *BcAPX*1-1, 1-8, 2-1, 3-2, and 3-5 had higher accumulations of the *BcAPX* protein than the other transgenic lines.

To prove that increasing heat resistance was conferred by introducing *BcAPX* into *Arabidopsis*, *BcAPX* cDNA was over-expressed in *Arabidopsis* under the control of the ubiquitin promoter. The APX activities in transgenic lines *BcAPX*1-1, 1-3, 1-4, 1-5, 1-8, 2-1, 2-3, 3-2, 3-3, and 3-5 were significantly higher than in the NT plants under both the non-heat and heat treatments. However, the APX activity of *BcAPX*2-2 did not significantly differ from the NT plants (Fig. 3). The *in vivo* detection of H_2O_2 in transgenic *Arabidopsis* was also observed and the fuscous precipitant appeared in the leaves of the NT plants under the heat stress treatment, whereas the transgenic *BcAPX*1-1, 1-3, 1-4, 1-5, 1-8, 2-1, 2-3, 3-2, 3-3, and 3-5 lines exhibited lower accumulations of H_2O_2 compared to the NT plants (Fig. 4).

The leaves of the NT plants visually exhibited epinasty, retardation, and a small size. However, in the transgenic *BcAPX*1-3, 2-1, and 3-5 plants, all leaves looked green, healthy, and large (Fig. 5), and some leaves looked green and healthy in the rest of the transgenic lines (data not shown). The heat stress had a harmful effect on the leaves of the NT plants. The transgenic lines *BcAPX*1-3, 2-1, and 3-5 were therefore used for the measurement of chlorophyll content, lipid peroxidation, and germination rate. The results show that the total chlorophyll content responded differently to the heat stress (Fig. 6*A*). Compared to NT, the chlorophyll content was significantly higher in *BcAPX*1-3, 2-1, and 3-5 exposed to the high temperature indicating that the extent of chlorophyll degradation was less in all the transgenic lines. However, there were no significant differences in chlorophyll content among the transgenic lines and the NT plants under the non-heat treatment. A significantly lower MDA content under the high temperature was observed in all the transgenic lines compared to the NT plants (Fig. 6*B*). MDA content in all the plants remained low and stable under the non-heat treatment. The germination rates of the NT and transgenic seeds grown under the non-heat treatment were approximately 100 %; however, the rates of the *BcAPX*1-3, 2-1, and 3-5 seeds (88, 91, and 92 %, respectively) subjected to the heat-stress treatment were significantly higher than in the NT seeds (56 %) (Fig. 6*C*).

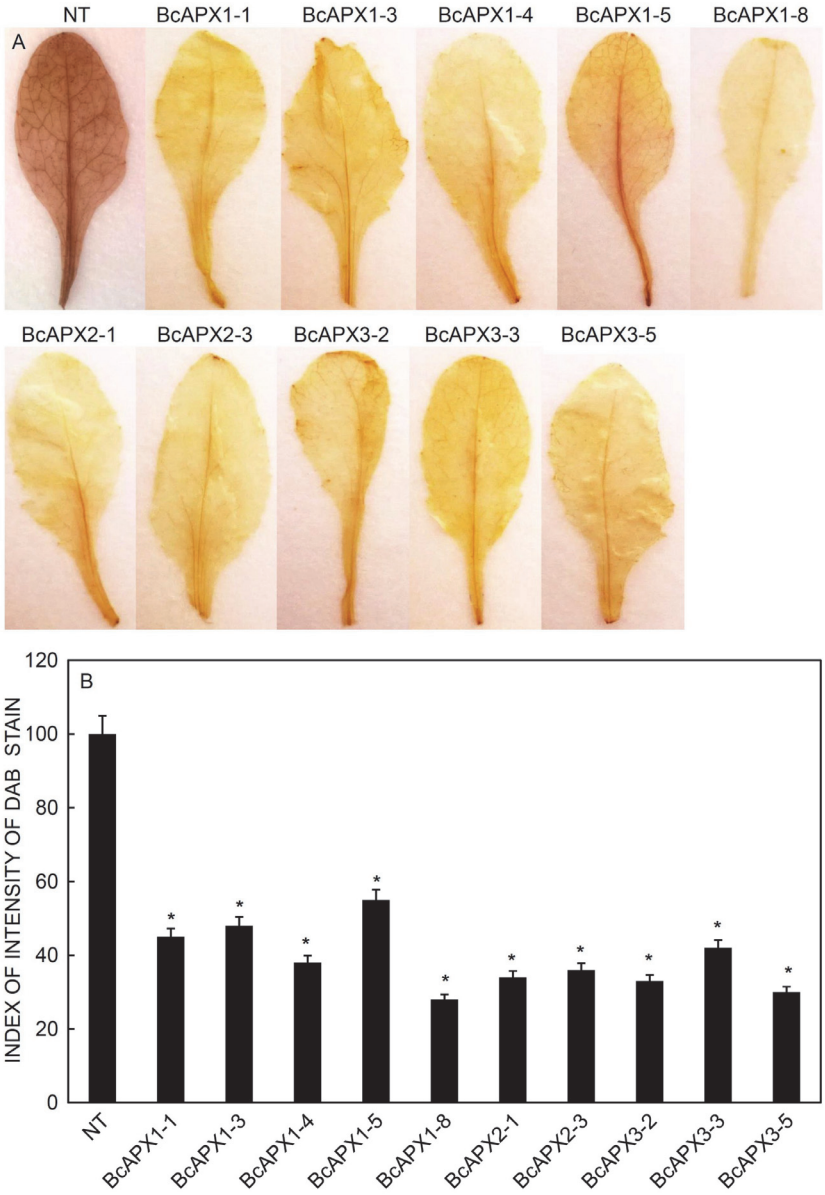

Fig. 4. H_2O_2 accumulation according to diaminobenzidine staining in NT and transgenic *Arabidopsis* lines after their exposure to 40 °C for 5 h (*A*) and its quantitative analysis (*B*). Means ± SD, $n = 3$,* - significant difference at $P \leq 0.05$ according to the *t*-test.

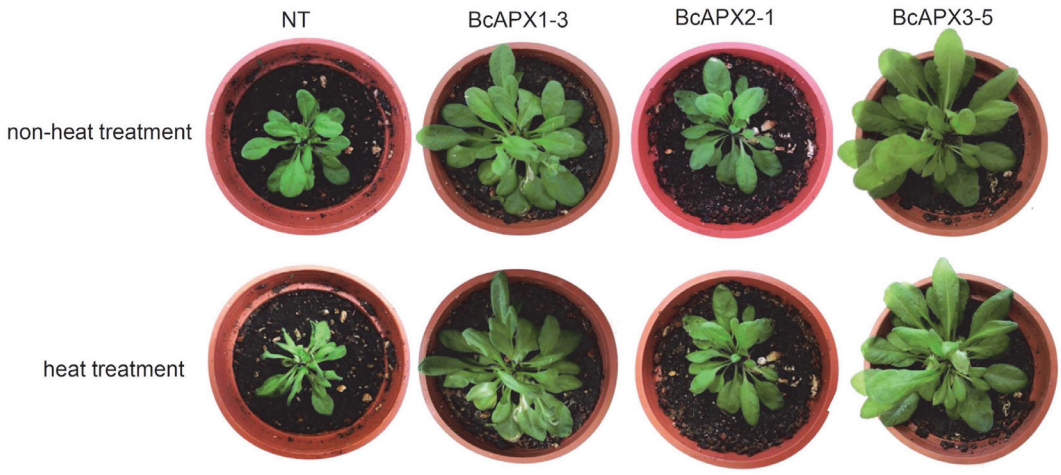

Fig. 5. The *in vivo* thermotolerance assay of NT and trasgenic *BcAPX*1-3, 2-1, and 3-5 plants grown at 22 °C for 21 d, then 3 d of a heat stress at 40 °C for 5 h, followed by recovery at 25/22 °C (day/night) for 2 h.

Discussion

The expression of *APX* was upregulated by the high-temperature treatment in ASVEG2 (Fig. 1*A*). Many reports indicated that potential signaling molecules, such as salicylic acid, abscisic acid, $CaCl_2$, H_2O_2, 1-amino-cyclopropane-1-carboxylate, and ROS, may reduce oxidative damage by activating the antioxidant system (Larkindale and Huang 2004, Montero-Barrientos *et al.* 2010, Wu *et al.* 2010, Ishikawa *et al.* 2013). To prove that an increasing heat resistance was conferred by introducing *BcAPXs* into *Arabidopsis*, *BcAPX* cDNAs were over-expressed in *Arabidopsis* under the control of the ubiquitin constitutive promoter. Different expressions in all the transgenic lines were observed, and the RNA abundance of *APX* was up-regulated after the high temperature stress (Fig. 1*B*). The high accumulation of the APX protein (Fig. 2) also suggested a high APX activity (Fig. 3) and was in correspondence with highly expressed *APX* in the *BcAPX*1-1, 1-8, 2-1, 3-2, and 3-5 transgenic lines (Fig. 1*B*). In contrast to the overexpressing lines, the RNA content in transgenic line *BcAPX*2-2 (Fig. 1*B*) was not translated into protein expression (Fig. 2). The low activity of APX in the 2-2 line (Fig. 3) might have been caused by post-transcriptional modifications, such as gene silencing by RNAi and SiRNA (Vaucheret *et al.* 2001, Lau and Korban 2009), due to a random insertion event of T-DNA (Kim *et al.* 2007). *APX* itself was transiently activated at the RNA level by the high temperature. APX might also respond to an oxidative stress caused by the heat stress at the post-translational level. Diaz-Vivancos *et al.* (2013) reported that transgenic tobacco plants over-expressing *SOD* and/or *APX* show an increased tolerance to drought stress by enhancing antioxidant defenses. Increases in the activity of some antioxidant enzymes was also observed in the chloroplast of these transgenic plants, suggesting a positive influence of antioxidant machinery in protecting the chloroplast and underlining the complexity of the regulation network of plant antioxidant defenses during stresses. Treating transgenic plant cells with the heat stress resulted in an alteration of *APX* transcription, and all of the *BcAPX* transcripts detected in the transgenic lines were significantly higher than in the NT plants except for the *BcAPX*2-2 line.

The DAB histochemical staining method is widely used to identify cellular H_2O_2 accumulation. A high H_2O_2 accumulation was observed as a brown colour precipitant in the NT plants under the heat stress (Fig. 4). The leaves of the transgenic plants maintained APX activities at a level, which decomposed at least part of the heat-induced H_2O_2 into water and oxygen (Fig. 3). ROS scavenging is important in imparting tolerance against heat stress (Lin *et al.* 2010). The *BcAPX* genes in the transgenic plants were involved in H_2O_2 detoxification and thus helped overcome the stress induced by heat. In addition, APX plays diverse roles in resistance to oxidative stress and possibly in mediating signal transduction involving H_2O_2 as second messenger (McClung 1997).

Plant growth was impaired under the high temperature in the NT plants, but less in the transgenic lines that exhibited a stronger heat tolerance due to a less oxidative injury (Fig. 5). The over-expression of *BcAPX* in the transgenic plants significantly increased heat tolerance compared to the NT plants. The *BcAPX* plants might use an APX-dependent mechanism to cope with the high temperature stress during seed germination (Fig. 6*C*) and overall plant growth (Fig. 5). Also Xi *et al.* (2010) demonstrated that the seed-specific over-expressions of *SOD* and *CAT* genes in *Arabidopsis* enhance oxidation stress tolerance during germination.

The amounts of the *APX* transcripts in the transgenic plants were higher than in the NT plants (Fig. 1*B*), which was consistent with the APX activity (Fig. 3) and

chlorophyll content (Fig. 6*A*). The transgenic lines showed a significantly lower MDA content than the NT plants under the high temperature (Fig. 6*B*) reflecting its tolerant nature. Heat stress causes membrane lipid peroxidation and aggravates membrane injury in many plant species (Song *et al.* 2012). Membrane and chloroplast damage under stress conditions is mainly caused by ROS generation (Djanaguiraman *et al.* 2010). The above mentioned transgenic plants overexpressing *APX* or *SOD* genes also exhibit less chlorophyll degradation and decreased lipid peroxidation in response to several abiotic stresses (Murgia *et al.* 2004, Lee *et al.*

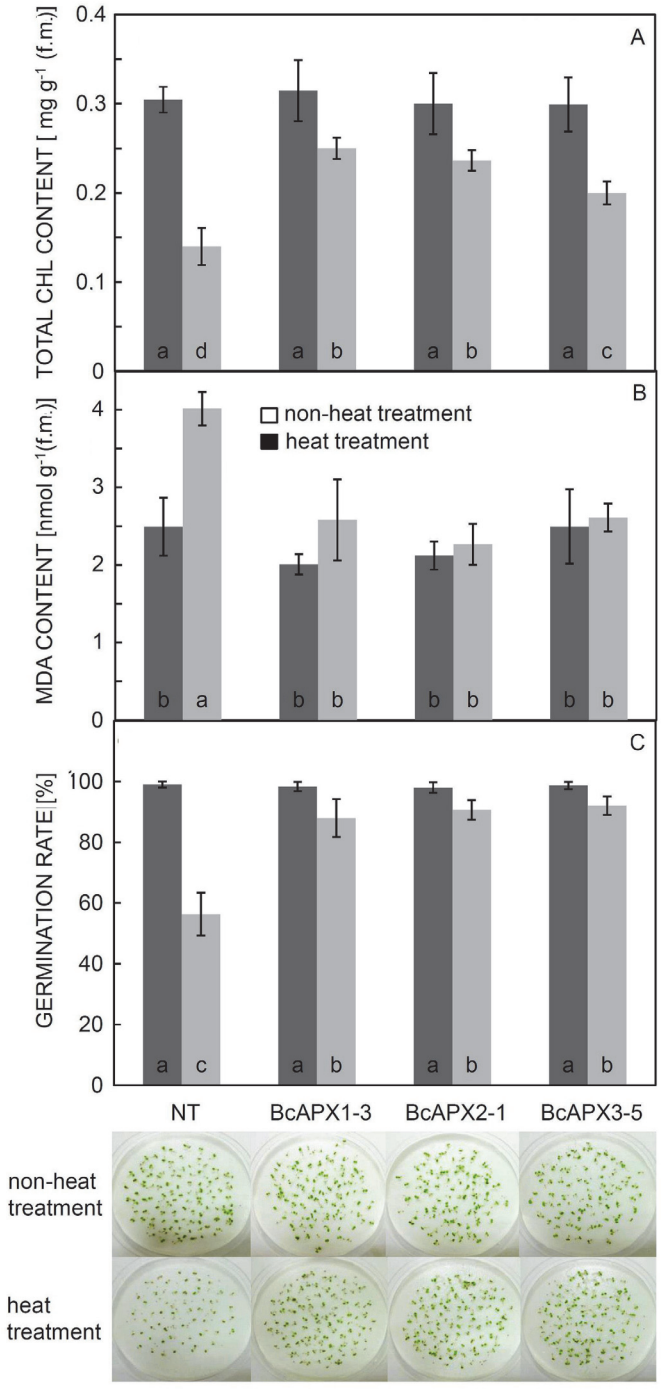

Fig. 6. The total chlorophyll content (*A*), malondialdehyde content (*B*), germination rate (*C*), and overview of seed germination of NT and transgenic *Bc*APX1-3, *Bc*APX2-1, and *Bc*APX3-5 plants after exposure to a heat stress (40 °C for 5 h) and a non-heat treatment. Six-day-old seedlings were used for the measurements immediately after stress. Means ± SD, $n = 3$. Means with the same lowercase letter do not significantly differ by the least significant difference (LSD) test.

2007b). The heat-inducible transcriptional activation of the *BcAPX* gene corresponded to an increase in APX activity in the cytosol to protect cellular components against the effects of ROS produced. *BcAPX* transgenic lines may provide information for selecting crop plants having better germination rates and heat tolerance. These findings are important for farming in areas under heat stress.

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