BROCCOLI PLANTS OVER-EXPRESSING A CYTOSOLIC ASCORBATE PEROXIDASE GENE INCREASE RESISTANCE TO DOWNY MILDEW AND HEAT STRESS

M. Jiang[1], J.J. Jiang[2], C.M. He[1] and M. Guan[1]

[1] *Ecology Key Discipline of Zhejiang Province, College of Life Science, Taizhou University, Jiaojiang 318000, China*
[2] *State Key Laboratory of Agrobiotechnology Shenzhen Base, Shenzhen Research Institute, The Chinese University of Hong Kong, Shenzhen 518057, China*

SUMMARY

Ascorbate peroxidase (APX) plays an important role in scavenging excessive reactive oxygen species (ROS) produced under environmental stresses, thus protects plant cells from oxidative injury. Seven *Brassica oleracea* var. *italica* (broccoli) lines over-expressing *BoAPX* gene were obtained using *Agrobacterium tumefaciens* transformation methods. The *BoAPX* over-expression plants exhibited significant higher resistance to *Hyaloperonospora parasitica* infection and heat stress as compared to the wild type broccoli. Among them, four gene over-expression lines, *oe-apx07*, *oe-apx15*, *oe-apx32* and *oe-apx33*, demonstrated extremely higher enhanced tolerance to downy mildew. In addition, when treated with either *H. parasitica* or high temperature, lower level of relative electrical conductivity and higher level of APX enzyme activity were both observed in the *oe-apx* lines. These results indicated that over-expression of *BoAPX* gene contributes enhanced tolerance to both downy mildew and heat stress, and *BoAPX* gene plays an essential role in cellular defense against ROS-mediated oxidative damage in broccoli.

Keywords: *Brassica oleracea* var. *italica*, ascorbate peroxidase, APX, downy mildew, heat stress, over-expression.

INTRODUCTION

Broccoli (*Brassica oleracea* var. *italica*), a member of Cruciferae family, is becoming more popular as a human diet of high nutritional value as well as a significant source of antioxidants, and its production and consumption have increased dramatically over the past decades (Zhang *et al*., 2004; Moreno *et al*., 2006). Broccoli contains rich fiber, potassium, calcium, vitamins, glucosinolates, carotenoids, flavonol glycosides and selenium, and plays important roles in reducing the risk of heart disease, diabetes and some cancers (Finley *et al*., 2001; Finley 2003; Matusheski *et al*., 2006; Mukherjee *et al*., 2008). Broccoli is an economically important core vegetable grown in more than 90 countries and consumed around the world (Chiang *et al*., 1998). Downy mildew, caused by *Hyaloperonospora parasitica* (formerly *Peronospora parasitica*), is a worldwide threat to broccoli production, which affects leaves, stems as well as flower heads, resulting in yield and market quality losses (Dickson and Petzoldt, 1993; Jiang *et al*., 2012a). Broccoli is a cool season crop, with the optimum mean temperature range from 18°C to 25°C (Lin *et al*., 2010), so besides the downy mildew, heat stress is also considered a threat to broccoli production, which causes rapid swelling of sepals, puffy buds, loose individual florets and leafy flower heads (Heather *et al*., 1992; Farnham and Bjorkman, 2011).

Both pathogen attack and heat stress induce production of reactive oxygen species (ROS) which include hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), singlet oxygen ($^1O_2$), and hydroxyl radicals ($OH^·$). Under normal conditions, ROS, generated as by-products of cellular metabolism, is necessary for cell proliferation, signaling, growth and development (Foreman *et al*., 2003; Mittler *et al*., 2011). However, excessive ROS will seriously disrupt normal plant metabolism by causing oxidation damage to membrane lipids, proteins, and nucleic acids (Fridovich *et al*., 1986; Rashad and Hussien, 2014). Fortunately, antioxidative defense mechanisms, including enzymatic and nonenzymatic antioxidants, have evolved for scavenging or detoxification of excess ROS under stress conditions (Hanukoglu *et al*., 2006). The enzymatic antioxidants include peroxidase (POD), ascorbic acid oxidase (AAO), polyphenol oxidase (PPO), catalase (CAT), guaiacol peroxidase (GPX), superoxide dismutase (SOD), ascorbate peroxidase (APX), and glutathione reductase (GR) (Noctor and Foyer, 1998; Blokhina *et al*., 2003; Gill and Tuteja, 2010; Caverzan *et al*., 2012; Sofo *et al*., 2015). Among them, APX (EC 1.11.1.11) is an important enzyme for metabolism of H_2O_2, and thus increases stability of membranes and prevents cells from injury (Yoshimura *et al*., 2000). Transgenic plants including *Nicotiana tabacum*, *Arabidopsis thaliana*, *Oryza sativa*, *Lycopersicon esculentum* and *B. napus* over-expressing *APX* genes were developed, and higher total APX activity than non-transgenic plants was detected (Shi

Corresponding author: M. Jiang
E-mail: jiangming1973@139.com

et al., 2001; Sarowar *et al*., 2005; Wang *et al*., 2005; Sato *et al*., 2011; Wang *et al*., 2011).

In our previous study, a cytosolic ascorbate peroxidase gene, designated *BoAPX* (GenBank accession No. HQ871864), was isolated from *B. oleracea* var. *italica*. *BoAPX* is orthologous to known *APX*s in *B. rapa* ssp. *pekinensis* (GQ500125), *B. napus* (Y11461) and *Raphanus sativus* (X78452). RT-PCR results indicated that the expression of *BoAPX* was induced by *Hyaloperonospora parasitica*, implying its probable function in downy mildew resistance. The gene was transferred into vector pBI121 driven by the constitutive cauliflower mosaic virus 35S promoter (CaMV 35S) with *npt*II as a selectable marker gene (Jiang *et al*., 2012b).

Downy mildew and high temperature are two major factors affecting plant growth and development. APX may play an increasingly important role in defending both biotic and abiotic stresses. Here we investigated the biological function of *BoAPX* during plant development. Transgenic lines over-expressing *BoAPX* were generated and the results indicated that over-expression of *BoAPX* gene increased tolerance to both downy mildew and heat stress, reflecting its possible function in cellular defense against ROS-mediated oxidative damage in broccoli.

MATERIALS AND METHODS

Plant material. A broccoli (*Brassica oleracea* var. *italica*) inbred line, Bo113, was used. The seeds were surface sterilized with 70% (v/v) ethanol solution for 5 min, and then soaked in 0.1% $HgCl_2$ for 6 min, followed by 5 brief rinses with sterile double-distilled water. The seeds were sowed on Murashige and Skoog (MS) medium, and were then cultured at 25 ± 1°C with a 16 h light and 8 h dark photoperiod in the plant growth room in the College of Life Science of Taizhou University.

***Agrobacterium* transformation.** The recombinant plasmid pBI121-*BoAPX* and empty vector PBI121 (control) were introduced into *Agrobacterium tumefaciens* strain LBA4404, respectively. For genetic transformation, stems from 15-day old seedlings were cut into 1.0 cm length segments and inoculated with *A. tumefaciens* cells carrying the recombinant plasmid. The pre- and co-culture mediums were MS supplemented with 0.02 mg/l of naphthaleneacetic acid (NAA), 4.0 mg/l of 6-benzylaminopurine (6-BA), and 5.0 mg/l of $AgNO_3$. The shoot induction medium was MS plus 0.02 mg/l of NAA, 4.0 mg/l of 6-BA, 4.0 mg/l of $AgNO_3$ and 50.0 mg/l of kanamycin. Shoots were rooted in MS containing both 0.2 mg/l of NAA and 50.0 mg/l of kanamycin (Jiang *et al*., 2012a).

PCR confirmation of transgenic plants. Genomic DNA was isolated by using CTAB method (Doyle and Doyle, 1987). Primer pairs of NPTUP (TGCTCGACGTTGTCACTG) and NPTDN (GCATCGCCATGGGTCAC) were designed to amplify a fragment of the selective marker gene *npt*II, and the primer pairs of BoUP1 (AGGACCTAACAGAACTCGC) and BoDN1 (CCAGGGTGGAAAGGAATCTCA) were used to amplify partial sequences of 35S promotor and *BoAPX* gene, respectively. PCR reaction mixture consisted of 30 ng of gDNA, 200 mM of each dNTP, 20 pmol primers, 1.2 U of Taq DNA polymerase (Promega, USA), 2 µl PCR buffer, and 40 mM $MgCl_2$ in total volume of 20 µl. PCR amplification was carried out in Bio-Rad C1000 Thermal Cycler with 32 cycles of 95°C (35 s), 50.5°C (45 s) and 72°C (60 s), and a final extension of 72°C for 10 min. PCR products were separated on 1.2% agarose gel containing 0.5 µg/ml ethidium bromide.

Disease assessment. Plants of seven *BoAPX* over-expression lines were propagated using stems as explants and transplanted into climatic chambers. Six plants of each line were used. The type of downy mildew strain used for the inoculation was Boihp23. Spore suspensions were prepared by washing the conidia off the leaf surface, and 0.2 ml of suspensions (approximately 1×10^5 spores per microliter) containing 0.01% (v/v) Tween 20 were sprayed onto each side of leaves. The control plants were treated with equal amount of ddH_2O with 0.01% (v/v) Tween 20. The plants were cultured in the cabinet at 16°C to maintain cool and damp (RH 80%) conditions with 16 h/8 h light/dark cycles. Leaf samples were harvested 0 DAI (days after inoculation), 1 DAI, 3 DAI and 5 DAI, and were then washed and dried on tissue paper. All samples were stored in –80°C for APX enzyme assays. Five days after inoculation, disease assessment was carried out by using a six-point (0, 1, 3, 5, 7, 9) scale in which zero corresponded to neither necrotic flecks nor sporulation on leaf surface, and 9 to evident necrosis and some chlorosis with uniformly heavy sporulation (Dickson and Petzoldt, 1993). Disease indices were calculated based on Williams' formula (Williams, 1985).

Heat stress treatments. Plants were grown in climatic cabinets at 80% relative humidity, with a photoperiod of 16 h/8 h (light/dark). Day/night temperature regime (40°C/35°C) was applied for the transgenic and control plants as the heat stress treatment. Three biological replicates were conducted for both treatments with each replicate of 30 plants. After 0 DAI, 1 DAI, 3 DAI and 5 DAI, leaf samples were collected, washed, and dried before electrical conductivity determination and APX enzyme measurement.

Enzyme activity assays. About 200 mg of leaves harvested from each treated plants were ground into fine powder in liquid nitrogen and then mixed with 10 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA, 1 mM ascorbate, 0.1% (w/v) PMSF (phenylmethanesulfonyl

fluoride), and 20% (w/v) sorbitol (Shi *et al.*, 2001). For each treatment, six plants were used. The homogenate was centrifuged at 13000 *g* and 4°C for 30 min, and the supernatants were collected for enzyme assays. The APX enzyme activity assay was performed in a reaction mixture containing 50 mM potassium phosphate (pH 7.0), 0.1 mM EDTA, 1 mM H_2O_2, 1.5 mM ascorbate, and 50 µl of the crude enzyme extracts. The value of absorption at 290 nm was recorded at 80 s after the addition of H_2O_2, and the concentration was calculated according to micromole of ascorbate oxidized per minute ($E = 2.8$ mM/cm) (Nakano and Asada, 1981; Lin *et al.*, 2010).

Electrical conductivity measurement. Six leaf discs of 6 mm diameter were punched out of each sample and immersed in test tubes with 15 ml distilled water, and then were placed in a 25°C water bath for 24 h. The initial conductivity (EC_1) was measured using a DDS-11A conductivity meter. The test tubes were then kept in a boiling water bath for 30 min, and cooled to 25°C for a final conductivity determination (EC_2). The relative EC value (%) was calculated as $EC_1 / EC_2 \times 100\%$ (Apostolova *et al.*, 2008).

Statistical analysis. Comparisons between wild type and transgenic broccoli plants were performed using one-way analysis of variance (ANOVA) and least significant difference (LSD) test (Tang and Zhang, 2013).

RESULTS

PCR detection of transgenic plants. To determine the biological function of the *BoAPX* gene, *BoAPX* driven by CaMV 35S promoter was transformed into broccoli wild type plants. A total of seven over-expression transgenic lines, namely *oe-apx07*, *oe-apx15*, *oe-apx22*, *oe-apx27*, *oe-apx32*, *oe-apx33* and *oe-apx49*, were screened out of 63 regenerated plantlets by using kanamycin, and they were later confirmed by PCR. Amplification products of the *npt*II gene were observed in both WT and transgenic plants, however, the fragments of partial 35S promotor and *BoAPX* were present only in those transgenic lines, and no band was observed in the WT line (Fig. 1).

Assessment of downy mildew resistance. Enough transgenic broccoli plants were generated using tissue culture method. Reaction phenotypes were assigned, and disease indices were calculated (Table 1). The control plants exhibited a susceptible reaction with disease index of 6.95, while the over-expression lines showed different resistance classes, from LR to VR. Necrotic lesions, chlorosis, and heavy sporulation were observed on leaves of wild type plants which was downy mildew susceptible as we identified previously. Necrotic lesions as well as sparse sporulation were presented on the leaves of *oe-apx22* with disease index of 5.10. *Oe-apx27* and *oe-apx49* were two moderately resistant lines with disease indices of 3.40 and 4.75, respectively, and necrotic flecks and few sporangiophores were observed on their leaves. Interestingly, four lines, *oe-apx07*, *oe-apx15*, *oe-apx32* and *oe-apx33*, showed a very high degree of resistance to downy mildew with disease indices of 2.94, 1.08, 1.79 and 2.94, respectively. No necrotic flecks or sporulation was detected on leaves in *oe-apx15* (Fig. 2).

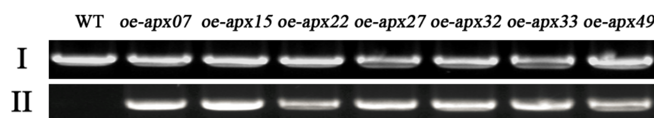

Fig. 1. PCR detection of transgenic plants. I: PCR detection of *NPT*II gene in both WT and transgenic lines; II: PCR detection of partial sequences of 35S promoter and *BoAPX* gene; WT: the control plant with empty vector; *oe-apx07*, *oe-apx15*, *oe-apx22*, *oe-apx27*, *oe-apx32*, *oe-apx33* and *oe-apx49*: *BoAPX* over-expression lines.

Table 1. Evaluation of downy mildew interaction–phenotype classes of broccoli transgenic lines.

Lines	Interaction–phenotype class[1]						Plant tested	Disease index[2]	Resistance class[3]
	0	1	3	5	7	9			
WT	–	–	–	27	44	25	96	6.95 Aa	S
oe–apx07	–	32	35	29	–	–	96	2.94 De	VR
oe–apx15	38	35	23	–	–	–	96	1.08 Fg	VR
oe–apx22	–	–	23	45	28	–	96	5.10 Bb	LR
oe–apx27	–	13	51	32	–	–	96	3.40 Cd	MR
oe–apx32	22	32	35	7	–	–	96	1.79 Ef	VR
oe–apx33	–	31	38	26	1	–	96	2.94 De	VR
oe–apx49	–	3	32	35	26	–	96	4.75 Bc	MR

[1] 0 = no necrotic flecks, no sporulation; 1 = small necrotic flecks, no sporulation; 3 = necrotic flecks, one to few sporangiophores; 5 = necrotic lesions, sparse scattered sporulation usually confined to necrotic areas; 7 = necrotic lesions, sometimes with accompanying chlorosis, scattered, heavy to abundant sporulation in both chlorotic and necrotic areas; 9 = necrosis and some chlorosis may be evident, uniformly heavy sporulation over abaxial surface of leaf.

[2] Values with different lowercase/uppercase letters are significantly different at $P < 0.05 / P < 0.01$ according to LSD's test, respectively.

[3] Resistance classes based on disease indices (DI) calculated by Williams' formula: VR (very resistant), DI = 0-3.0; MR (moderately resistant), DI = 3.1-5.0; LR (low resistance), DI = 5.1-6.0; S (susceptible), DI = 6.1-7.0; VS (very susceptible), DI = 7.1-9.0.

Leaves of WT and seven over-expression lines were used for APX enzyme activity assay. Comparing with the control plants, the APX enzyme activity were higher in all the *BoAPX* over-expression lines at 0 DAI, which were probably be due to *BoAPX* over-expression. When challenged with *H. parasitica*, both the control and over-expression plants exhibited increased APX enzyme activity at 1 and 3 DAI, and decreased at 5 DAI. Compared with the control plants, all the over-expression lines showed higher enzyme activity at 1 and 3 DAI. A maximal level

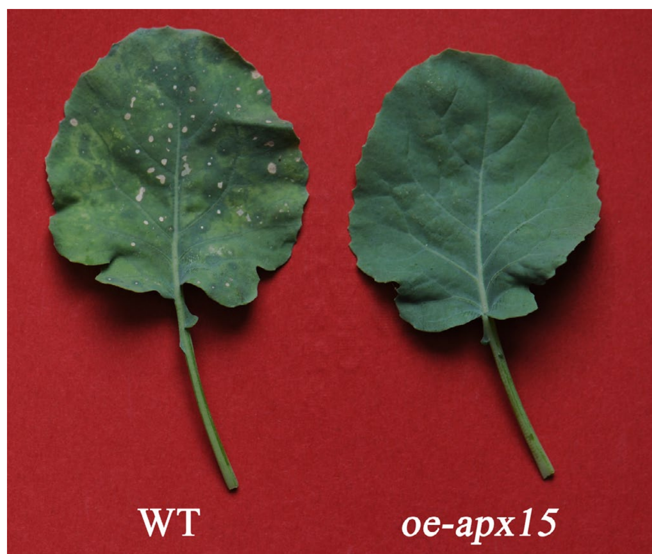

Fig. 2. *Hyaloperonospora parasitica* lesions on leaves of control and *BoAPX* over-expression plants. WT: The leaf from control plants, necrosis and some chlorosis are evident on leaf surface; *oe-apx15*: a leaf of the *BoAPX* over-expression lines 15.

was observed at 3 DAI in line *oe-apx15* with the value at 15.10 ± 0.23 U/g FW, which was twice more than the control at the same time point (Table 2).

Assessment of electrical conductivity. The leaves of the control and over-expression plants were sprayed with *H. parasitica* and prepared for electrical conductivity (REC) measurement. REC values were identified from 0 d to 5 d after spray. Levels of REC in all samples increased at different ratio during pathogen inoculations. There were significant differences between the control and the seven lines at 0 DAI, and the highest REC was observed in control plants with value of 21.00 ± 0.61. The control plants challenged with *H. parasitica* exhibited the highest REC (73.45 ± 4.98) at 5 DAI, followed by *oe-apx07* (54.74 ± 2.11) and *oe-apx27* (51.30 ± 1.21). However, REC of both *oe-apx07* and *oe-apx27* were much lower than that of the control plants. The RECs at 5 DAI were relative lower in*oe-apx15* (43.79 ± 1.24), *oe-apx22* (42.55 ± 0.63), *oe-apx32* (44.70 ± 2.25), *oe-apx33* (42.66 ± 0.90) and *oe-apx49* (44.18 ± 1.40), with no significant differences among them (Table 3).

Assessment of APX enzyme activity under heat resistance. The APX enzyme activity under heat stress was assayed in leave in the *oe-apx* and wt lines. All the *oe-apx* lines showed significantly higher APX enzyme activity than the control at 0 DAH and the highest value was observed for *oe-apx33*, with enzyme activity of 7.70 ± 0.27. Under heat stress, all transgenic plants exhibited increased APX enzyme activity at 1 DAI and 3 DAI except for *oe-apx07*, it decreased at 3 DAI with enzyme activity of 7.81 ± 0.14. Compared to control broccoli, all the transgenic evidenced higher enzyme activity during heat stress except for *oe-apx22* line, its APX activity (7.55 ± 0.39 U/g FW) had no significant difference with that of control at 1 DAI. The highest value of transgenic broccoli was detected at 3 DAI in the *oe-apx07* line, with enzyme activity of 12.05 ± 0.51 U/g FW. The lowest value during heat stress was 6.63 ± 0.43 in *oe-apx49* line, which was more than 1.5-fold enzyme activity as compared to the control (Table 4).

REC was identified in leaves of control and over-expression broccoli plants under heat stress. Significant differences were observed among the lines (Table 5). The highest and lowest RECs were identified in WT and *oe-apx15* plants at 0 DAH, with values at 20.97 ± 0.62 and 16.96 ± 0.58, respectively. Subjected to heat stress, REC in all leaf samples increased to different ratio. *Oe-apx15* line showed minimum electrolyte leakage after 5 DAH. Furthermore, the other six over-expression lines also demonstrated lower REC (less than 50%) as compared to the control plants, indicating less cell membrane damage in *BoAPX* over-expression lines than the control plants when exposed to heat stress (Table 5).

DISCUSSION

Plants are often subjected to multiple abiotic and biotic environmental stresses. They must acquire greater tolerance to maintain normal growth, development and productivity. Metabolic activities in various cellular compartments lead to the production of ROS (Shafi *et al*., 2014). Normally, moderate levels of ROS may function as signals to promote cell proliferation and survival, however, under severe stress condition, overproduced ROS attacks lipids, proteins, and DNA, leading to serious and irreversible damage to cells (Trachootham *et al*., 2008). APX enzymes, serving as electron donors in reactions, are effective antioxidant molecules for eliminating excessive ROS, thus protect cells from injury by toxic levels of ROS (Narendra *et al*., 2006; Anjum *et al*., 2014).

APX isoenzymes are distributed in various cellular compartments as cytoplasm, chloroplast and microbody. There are eight members in *Arabidopsis*, these enzymes belong to stromal APX (sAPX), thylakoid membrane-bound APX (tAPX), microbody (including glyoxysome and peroxisome) membrane-bound APX (mAPX), and cytosolic APX (cAPX) (Panchuk *et al*., 2005; Shigeoka *et al*., 2014). Among them, cAPX enzymes are involved in multiple stress responses, and can be activated by ozone, sulfur dioxide, pathogen, excessive light, heat and heavy metal stress (Mittler *et al*., 1999; Shigeoka *et al*., 2002; Panchuk *et al*., 2005). In this study, a *cAPX* gene designated *BoAPX* was over-expressed in broccoli under the control of CaMV 35S promoter, and a total of seven over-expression lines were obtained and used for downy mildew infection and heat stress response assay.

Table 2. Effect of *Hyaloperonospora parasitica* on APX enzyme activity in leaves of transgenic broccoli plants (U/g FW).

Broccoli lines	Days after inoculation[1]			
	0 d	1 d	3 d	5 d
WT	5.13±0.12 Cc	6.36±0.35 Gg	6.63±0.32 Ff	4.52±0.34 Dc
oe-apx07	7.54±0.43 Aa	10.74±0.47 Bb	12.15±0.93 Cc	7.41±0.11 Cb
oe-apx15	7.30±0.17 Aa	12.50±0.42 Aa	15.10±0.23 Aa	7.70±0.41 BCb
oe-apx22	6.36±0.27 Bb	7.10±0.34 FGf	8.66±0.12 Ee	7.52±0.28 Cb
oe-apx27	7.71±0.26 Aa	8.54±0.38 DEd	10.33±0.21 Dd	8.34±0.19 ABa
oe-apx32	7.63±0.21 Aa	9.33±0.28 CDc	13.45±0.29 Bb	8.72±0.22 Aa
oe-apx33	7.63±0.41 Aa	9.74±0.49 Cc	12.04±0.45 Cc	8.29±0.45 ABa
oe-apx49	6.58±0.19 Bb	7.81±0.22 EFe	9.58±0.41 DEd	7.52±0.25 Cb

[1] Mean values±standard errors are shown (n=6).
Different lowercase and uppercase letters indicate significant differences among broccoli lines at $p<0.05$ and $p<0.01$, respectively.

Table 3. The relative electrical conductivity (%) change in leaves after spray of *Hyaloperonospora parasitica* in the over-expression and control plants of broccoli.

Broccoli lines	Days after inoculation[1]			
	0 d	1 d	3 d	5 d
WT	21.00±0.61 Aa	34.18±2.35 Aa	56.34±1.94 Aa	73.45±4.98 Aa
oe-apx07	17.89±0.56 BCbcd	27.95±1.00 Bb	42.64±0.44 Bb	54.74±2.11 Bb
oe-apx15	16.97±0.58 Cd	20.17±1.42 Dd	27.05±1.23 Ee	43.79±1.24 Cc
oe-apx22	18.70±0.50 Bb	27.54±1.25 BCb	38.36±1.69 Cc	42.55±0.63 Cc
oe-apx27	17.96±0.46 BCbcd	24.53±1.38 Cc	33.89±1.52 Dd	51.30±1.21 Bb
oe-apx32	17.22±0.62 Ccd	21.15±0.73 Dd	31.81±1.15 Dd	44.70±2.25 Cc
oe-apx33	17.92±0.75 BCbcd	32.93±1.43 Aa	33.15±0.84 Dd	42.66±0.90 Cc
oe-apx49	18.06±0.61 BCbc	27.07±0.34 BCb	33.55±1.30 Dd	44.18±1.40 Cc

[1] Mean values±standard errors are shown (n=6).
Different lowercase and uppercase letters indicate significant differences among broccoli lines at $p<0.05$ and $p<0.01$, respectively.

Table 4. The assessment of APX enzyme activity in broccoli leaves under heat stress (U/g FW).

Broccoli lines	Days after heat stress[1]			
	0 d	1 d	3 d	5 d
WT	5.17±0.16 Bc	7.27±0.17 Ed	5.47±0.35 Ee	4.41±0.30 De
oe-apx07	7.46±0.40 Aab	10.53±0.39 Aa	12.05±0.51 Aa	8.67±0.62 Aab
oe-apx15	7.70±0.27 Aa	9.22±0.21 Bb	11.56±0.39 Aa	7.43±0.18 BCc
oe-apx22	6.58±0.40 Ab	7.55±0.39 DEd	9.37±0.27 BCb	8.47±0.44 ABab
oe-apx27	7.40±0.42 Aab	8.51±0.42 BCc	9.45±0.38 BCb	9.16±0.41 Aa
oe-apx32	7.63±0.44 Aa	8.29±0.33 CDc	8.57±0.31 CDc	8.13±0.51 ABbc
oe-apx33	7.41±0.23 Aab	8.55±0.39 BCc	9.51±0.43 Bb	7.44±0.39 BCc
oe-apx49	6.76±0.10 Aab	8.45±0.29 BCc	7.81±0.14 Dd	6.63±0.43 Cd

[1] Mean values±standard errors are shown (n=6).
Different lowercase and uppercase letters indicate significant differences among broccoli lines at $p<0.05$ and $p<0.01$, respectively.

Table 5. The relative electrical conductivity (%) change in leaves under heat stress in the over-expression and control plants of broccoli.

Broccoli lines	Days after heat stress[1]			
	0 d	1 d	3 d	5 d
WT	20.97±0.62 Aa	28.02±0.77 ABab	44.40±0.79 Aa	69.70±0.45 Aa
oe-apx07	17.83±0.65 BCbcd	24.21±0.96 CDcd	37.49±1.35 Cc	45.32±0.87 Bb
oe-apx15	16.96±0.58 Cd	23.36±0.55 Dd	27.36±0.54 Ff	39.23±0.61 Ef
oe-apx22	18.67±0.68 Bb	30.36±1.35 Aa	34.46±1.36 Dd	43.08±0.11 Cc
oe-apx27	17.89±0.59 BCbcd	26.32±1.68 BCbc	34.22±0.34 Dd	41.82±0.56 CDde
oe-apx32	17.19±0.61 BCcd	22.95±0.58 Dd	39.67±0.58 Bb	42.17±0.95 CDcd
oe-apx33	17.88±0.52 BCbcd	29.56±0.40 Aa	34.83±0.85 Dd	42.15±0.56 CDcde
oe-apx49	18.15±0.55 BCbc	22.55±0.94 Dd	31.10±0.87 Ee	40.92±0.93 DEe

[1] Mean values±standard errors are shown (n=6).
Different lowercase and uppercase letters indicate significant differences among broccoli lines at $p<0.05$ and $p<0.01$, respectively.

Environmental stresses disrupt metabolic balance of cells, resulting in enhanced production of ROS. Detoxification of ROS needs more antioxidants. Over-expression of *APX* genes increases APX enzyme activity and improves ROS scavenge ability. Over-expression of *LetAPX* enhanced resistance to chilling stress in tomato (*Lycopersicon esculentum*) (Duan *et al*., 2012). A *cAPX* gene from pea (*Pisum sativum*) was induced into tomato. The transformed lines were proved to be chilling and salt stress tolerant, and the APX activity in transgenic plants was several times higher than that in the control plants (Wang *et al*., 2005). Over-expression of a *Lycium chinense cAPX* gene in tobacco (*Nicotiana tabacum* cv. *SR-1*) showed higher APX activity and enhanced salt tolerance (Wu *et al*., 2014). In our study, significant higher level of APX enzyme activity was detected in over-expression lines, and all the transformed broccoli plants demonstrated increasing disease resistance and heat tolerance. Whether under downy mildew inoculation/heat stress or not, APX activity was much higher in transgenic lines than that in WT plants. In addition, different enzyme activity in the over-expressing plants were observed when challenged by downy mildew or high temperature, especially in *oe-apx07* and *oe-apx15* lines which implied downy mildew and heat stress caused ROS accumulation in plants, and *BoAPX* over-expression improved ROS scavenging ability (Guan *et al*., 2015).

REC is regarded as an effective indicator for cellular membrane injury caused by oxidative stress, during exposure to unfavorable environmental conditions, deterioration of lipids, proteins as well as nucleic occur, causing increased leakage of solutes through the membrane (Shehab *et al*., 2010). However, plants over-expressing *APX* gene, are more resistant to abiotic or biotic by scavenging excessive ROS, thus improve stress tolerance. *A. thaliana* transformed with a *Rheum austral APX* gene exhibited higher tolerance to cold stress and lower REC, demonstrating less membrane damage during cold stress in transgenic plants (Shafi *et al*., 2014). A tomato antisense tAPX was introduced into tobacco, resulting in lower APX activities and higher relative electrical conductivity as compared to WT plants under methylviologen-mediated oxidative stress (Sun *et al*., 2013). With the exception of lines oe-apx22 (heat stress at 1 DAI) and oe-apx33 (both downy mildew and heat stress at 1 DAI), significant lower REC was observed in *APX* over-expression broccoli lines compared to those in control plants, irrespective of whether the plants were exposed to stresses or not. This finding is similar to that of APX activity in our present study, suggesting over-expression of *BoAPX* resulted in reduced REC and conferred stress tolerance in broccoli. Though remarkably differences were observed in both APX activity and REC under non-stress environment, growth and development were similar for both over-expressing lines and WT plants, suggesting *BoAPX* might not play a significant role under normal conditions. These results agreed with tobacco lines over-expressing a thylakoid *APX* named *JctAPX*, which displayed no morphological abnormalities compared to control plants (Liu *et al*., *2013*).

In conclusion, our results indicated that over-expression of *BoAPX* gene increased tolerance to both downy mildew and heat stress, and *BoAPX* gene might play an essential role in cellular defense against ROS-mediated oxidative damage in broccoli.

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REFERENCES

Anjum N.A., Gill S.S., Gill R., Hasanuzzaman M., Duarte A.C., Pereira E., Ahmad I., Tuteja R., Tuteja N., 2014. Metal/metalloid stress tolerance in plants: role of ascorbate, its redox couple, and associated enzymes. *Protoplasma* **251**: 1265-1283.

Apostolova P., Yordanova R., Popova L., 2008. Response of antioxidative defence system to low temperature stress in two wheat cultivars. *General And Applied Plant Physiology* **3-4**: 281-294.

Blokhina O., Virolainen E., Fagerstedt K.V., 2003. Antioxidants, oxidative damage and oxygen deprivation stress: a review. *Annals of Botany* **91**: 179-194.

Caverzan A., Passaia G., Rosa S.B., Ribeiro C.W., Lazzarotto F., Margis-Pinheiro M., 2012. Plant responses to stresses: Role of ascorbate peroxidase in the antioxidant protection. *Genetics and Molecular Biology* **35**: 1011-1019.

Chiang W.C.K., Pusateri D.J., Leitz R.E.A., 1998. Gas Chromatography/Mass Spectrometry Method for the determination of sulforaphane and sulforaphane nitrile in broccoli. *Journal of Agricultural and Food Chemistry* **46**: 1018-1021.

Dickson M.H., Petzoldt R., 1993. Plant age and isolate source affect expression of downy mildew resistance in broccoli. *HortScience* **28**: 730-731.

Doyle J.J., Doyle J.L., 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* **19**: 11-15.

Duan M., Feng H.L., Wang L.Y., Li D., Meng Q.W., 2012. Overexpression of thylakoidal ascorbate peroxidase shows enhanced resistance to chilling stress in tomato. *Journal of Plant Physiology* **169**: 867-877.

Farnham M.W., Bjorkman T., 2011. Evaluation of experimental broccoli hybrids developed for summer production in the eastern United States. *HortScience* **46**: 858-863.

Finley J.W., Ip C., Lisk D.J., Davis C.D., Hintze K.J., Whanger P.D., 2001. Cancer-protective properties of high-selenium broccoli. *Journal of Agricultural and Food Chemistry* **49**: 2679-2683.

Finley J.W., 2003. Reduction of cancer risk by consumption of selenium-enriched plants: enrichment of broccoli with selenium increases the anticarcinogenic properties of broccoli. *Journal of Medicinal Food* **6**: 19-26.

Foreman J., Demidchik V., Bothwell J.H., Mylona P., Miedema H., Torres M.A., Linstead P., Costa S., Brownlee C., Jones J.D., Davies J.M., Dolan L., 2003. Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. *Nature* **422**: 442-446.

Fridovich I., 1986. Biological effects of the superoxide radical. *Archives of Biochemistry and Biophysics* **247**: 1-11.

Gill S.S., Tuteja N., 2010. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiology and Biochemistry* **48**: 909-930.

Guan Q., Wang Z., Wang X., Takano T., Liu S., 2015. A peroxisomal APX from *Puccinellia tenuiflora* improves the abiotic stress tolerance of transgenic *Arabidopsis thaliana* through decreasing of H_2O_2 accumulation. *Journal of Plant Physiology* **175**: 183-191.

Hanukoglu I., 2006. Antioxidant protective mechanisms against reactive oxygen species (ROS) generated by mitochondrial P450 systems in steroidogenic cells. *Drug Metabolism Reviews* **38**: 171-196.

Heather D.W., Sieczka J.B., Dickson M.H., Wolfe D.W., 1992. Heat tolerance and holding ability in broccoli. *Journal of the American Society for Horticultural Science* **117**: 887-892.

Jiang M., Miao L.X., He C.M., 2012a. Overexpression of an oil radish superoxide dismutase gene in broccoli confers resistance to downy mildew. *Plant Molecular Biology Reporter* **30**: 966-972.

Jiang M., Miao L.X., Qian B.Y., 2012b. Cloning and expression analysis of ascorbate peroxidase gene BoAPX from *Brassica oleracea* var. *italica*. *Journal of Zhejiang University (Science Edition)* **39**: 345-351.

Lin K.H., Huang H.C., Lin C.Y., 2010. Cloning, expression and physiological analysis of broccoli catalase gene and Chinese cabbage ascorbate peroxidase gene under heat stress. *Plant Cell Reports* **29**: 575-593.

Liu Z., Bao H., Cai J., Han J., Zhou L., 2013. A novel thylakoid ascorbate peroxidase from Jatrophacurcas enhances salt tolerance in transgenic tobacco. *International Journal of Molecular Sciences* **15**: 171-185.

Matusheski N.V., Swarup R., Juvik J.A., Mithen R., Bennett M., Jeffery E.H., 2006. Epithiospecifier protein from broccoli (*Brassica oleracea* L. ssp *italica*) inhibits formation of the anticancer agent sulforaphane. *Journal of Agricultural and Food Chemistry* **54**: 2069-2076.

Mittler R., Herr E.H., Orvar B.L., van Camp W., Willekens H., Inzé D., Ellis B.E., 1999. Transgenic tobacco plants with reduced capability to detoxify reactive oxygen intermediates are hyperresponsive to pathogen infection. *Proceedings of the National Academy of Sciences, USA* 96: 14165-14170.

Mittler R., Vanderauwera S., Suzuki N., Miller G., Tognetti V.B., Vandepoele K., Gollery M., Shulaev V., Van Breusegem F., 2011. ROS signaling: the new wave?. *Trends in Plant Science* **16**: 300-309.

Moreno D.A., Carvajal M., López-Berenguer C., García-Viguera C., 2006. Chemical and biological characterisation of nutraceutical compounds of broccoli. *Journal of Pharmaceutical and Biomedical Analysis* **41**: 1508-1522.

Mukherjee S., Gangopadhyay H., Das D.K., 2008. Broccoli: a unique vegetable that protects mammalian hearts through the redox cycling of the thioredoxin superfamily. *Journal of Agricultural and Food Chemistry* **56**: 609-617.

Nakano Y., Asada K., 1981. Hydrogen Peroxide is Scavenged by Ascorbate-specific Peroxidase in Spinach Chloroplasts. *Plant Cell Physiology* **22**: 867-880.

Narendra S., Venkataramani S., Shen G., Wang J., Pasapula V., Lin Y., Kornyeyev D., Holaday A.S., Zhang H., 2006. The *Arabidopsis* ascorbate peroxidase 3 is a peroxisomal membrane-bound antioxidant enzyme and is dispensable for *Arabidopsis* growth and development. *Journal of Experimental Botany* **57**: 3033-3042.

Noctor G., Foyer C.H., 1998. Ascorbate and glutathione: keeping active oxygen under control. *Annual Review of Plant Physiology and Plant Molecular Biology* **49**: 249-279.

Panchuk I.I., Zentgraf U., Volkov R.A., 2005. Expression of the Apx gene family during leaf senescence of *Arabidopsis thaliana*. *Planta* **222**: 926-932.

Rashad R.T., Hussien R.A., 2014. A comparison study on the effect of some growth regulators on the nutrients content of maize plant under salinity conditions. *Annals of Agricultural Sciences* **59**: 89-94.

Sarowar S., Kim E.N., Kim E.N., Kim Y.J., Ok S.H., Kim K.D., Hwang B.K., Shin J.S., 2005. Overexpression of a pepper ascorbate peroxidase-like 1 gene in tobacco plants enhances tolerance to oxidative stress and pathogens. *Plant Science* **169**: 55-63.

Sato Y., Masuta Y., Saito K., Murayama S., Ozawa K., 2011. Enhanced chilling tolerance at the booting stage in rice by transgenic overexpression of the ascorbate peroxidase gene, OsAPXa. *Plant Cell Reports* **30**: 399-406.

Shafi A., Dogra V., Gill T., Ahuja P.S., Sreenivasulu Y., 2014. Simultaneous over-expression of PaSOD and RaAPX in transgenic *Arabidopsis thaliana* confers cold stress tolerance through increase in vascular lignifications. *PLoS One* **9**: e110302.

Shehab G.G., Ahmed O.K., El-beltagi H.S., 2010. Effects of various chemical agents for alleviation of drought stress in rice plants (*Oryza sativa* L.). *Notulae Botanicae Horti Agrobotanici Cluj-Napoca* **38**: 139-148.

Shi W.M., Muramoto Y., Ueda A., Takabe T., 2001. Cloning of peroxisomal ascorbate peroxidase gene from barley and enhanced thermotolerance by overexpressing in *Arabidopsis thaliana*. *Gene* **273**: 23-27.

Shigeoka S., Ishikawa T., Tamoi M., Miyagawa Y., Takeda T., Yabuta Y., Yoshimura K., 2014. Simultaneous over-expression of PaSOD and RaAPX in transgenic *Arabidopsis thaliana* confers cold stress tolerance through increase in vascular lignifications. *PLoS One* **9**: e110302.

Shigeoka S., Ishikawa T., Tamoi M., Miyagawa Y., Takeda T., Yabuta Y., Yoshimura K., 2002. Regulation and function of ascorbate peroxidase isoenzymes. *Journal of Experimental Botany* **53**: 1305-1319.

Sofo A., Scopa A., Nuzzaci M., Vitti A., 2015. Ascorbate Peroxidase and Catalase Activities and Their Genetic Regulation in Plants Subjected to Drought and Salinity Stresses. *International Journal of Molecular Science* **16**: 13561-13587.

Sun W.H., Wang Y., He H.G., Li X., Song W., Du B., Meng Q.W., 2013. Reduction of methylviologen-mediated oxidative stress tolerance in antisense transgenic tobacco seedlings through restricted expression of StAPX. *Journal of Zhejiang University SCIENCE B* **14**: 578-585.

Tang Q.Y., Zhang C.X., 2013. Data Processing System (DPS) software with experimental design, statistical analysis and data mining developed for use in entomological research. *Insect Science* **20**: 254-260.

Trachootham D., Lu W., Ogasawara M A., Nilsa R.D., Huang P., 2008. Redox regulation of cell survival. *Antioxid Redox Signal* **10**: 1343-1374.

Wang J.M., Fan Z.Y., Liu Z.B., Xiang J.B., Chai L., Li X.F., Yang Y., 2011. Thylakoid-bound ascorbate peroxidase increases resistance to salt stress and drought in *Brassica napus*. *African Journal of Biotechnology* **10**: 8039-8045.

Wang Y., Wisniewski M., Meilan R., Cui M., Webb R., Fuchigami L., 2005. Overexpression of cytosolic ascorbate peroxidase in tomato confers tolerance to chilling and salt stress. *Journal of the American Society for Horticultural Science* **130**: 167-173.

Williams P.H., 1985. *Crucifer genetics cooperative resource book*. Madison: University of Wisconsin.

Wu G., Wang G., Ji J., Gao H., Guan W., Wu J., Guan C., Wang Y., 2014. Cloning of a cytosolic ascorbate peroxidase gene from *Lycium chinense* Mill. and enhanced salt tolerance by overexpressing in tobacco. *Gene* **543**: 85-92.

Yoshimura K., Yabuta Y., Ishikawa T., Shigeoka S., 2000. Expression of spinach ascorbate peroxidase isoenzymes in response to oxidative stresses. *Plant Physiology* **123**: 223-234.

Zhang D.L., Hamauzu Y., 2004. Phenolics, ascorbic acid, carotenoids and antioxidant activity of broccoli and their changes during conventional and microwave cooking. *Food Chemistry* **88**: 503-509.

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