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

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SUMMARY

Ascorbate pero dase (APX) p ays an mportant ro e n scaveng ng e cess ve react ve o ygen spec es (ROS) produced under env ronmenta stresses, thus protects p ant ce s rom o dat ve njury. Seven *Brassica oleracea* var. *italica* (brocco) nes over-e press ng *BoAPX* gene were obta ned us ng *Agrobacterium tumefaciens* trans ormat on methods. The *BoAPX* over-e press on p ants e h b ted s gn cant h gher res stance to *Hyaloperonospora parasitica* n ect on and heat stress as compared to the w d type brocco . Among them, our gene over-e press on nes, *oe-apx07*, *oe-apx15*, *oe-apx32* and *oe-apx33*, demonstrated e treme y h gher enhanced to erance to downy m dew. In add t on, when treated w th e ther *H. parasitica* or h gh temperature, ower eve o re at ve e ectr ca conduct v ty and h gher eve o APX enzyme act v ty were both observed n the *oe-apx* nes. These resu ts nd cated that over-e press on o *BoAPX* gene contr butes enhanced to erance to both downy m dew and heat stress, and *BoAPX* gene p ays an essent a ro e n ce u ar de ense aga nst ROS-med ated o dat ve damage n brocco .

Keywords: *Brassica oleracea* var. *italica*, ascorbate pero dase, APX, downy m dew, heat stress, over-e press on.

INTRODUCTION

Brocco (*Brassica oleracea* var. *italica*), a member o Cruc erae am y, s becom ng more popu ar as a human d et o h gh nutr t ona va ue as we as a s gn cant source o ant o dants, and ts product on and consumpt on have ncreased dramat ca y over the past decades (Zhang *et al*., 2004; Moreno *et al*., 2006). Brocco conta ns r ch ber, potass um, ca c um, v tam ns, g ucos no ates, caroteno ds, avono g ycos des and se en um, and p ays mportant ro es n reduc ng the r s o heart d sease, d abetes and some cancers (F n ey *et al*., 2001; F n ey 2003; Matushes *et al*., 2006; Mu herjee *et al*., 2008). Brocco s an econom ca y mportant core vegetab e grown n more than 90 countr es and consumed around the wor d (Ch ang *et al*., 1998). Downy m dew, caused by *Hyaloperonospora parasitica* (ormer y *Peronospora parasitica*), s a wor dw de threat to brocco product on, wh ch a ects eaves, stems as we as ower heads, resu t ng n y e d and mar et qua ty osses (D c son and Petzo dt, 1993; J ang *et al*., 2012a). Brocco s a coo season crop, w th the opt mum mean temperature range rom 18°C to 25°C (L n *et al*., 2010), so bes des the downy m dew, heat stress s a so cons dered a threat to brocco product on, wh ch causes rap d swe ng o sepa s, pu y buds, oose nd v dua orets and ea y ower heads (Heather *et al*., 1992; Farnham and Bjor man, 2011).

Both pathogen attac and heat stress nduce product on o react ve o ygen spec es (ROS) wh ch nc ude hydrogen pero de (H_2O_2), supero de an on (O_2^-), s ng et o ygen ($^1O_2$), and hydro y rad ca s ($OH^·$). Under norma cond t ons, ROS, generated as by-products o ce u ar metabo sm, s necessary or ce pro erat on, s gna ng, growth and deve opment (Foreman *et al*., 2003; M tt er *et al*., 2011). However, e cess ve ROS w ser ous y d srupt norma p ant metabo sm by caus ng o dat on damage to membrane p ds, prote ns, and nuc e c ac ds (Fr dov ch *et al*., 1986; Rashad and Huss en, 2014). Fortunate y, ant o dat ve de ense mechan sms, nc ud ng enzymat c and nonenzymat c ant o dants, have evo ved or scaveng ng or deto cat on o e cess ROS under stress cond t ons (Hanu og u *et al*., 2006). The enzymat c ant o dants nc ude pero dase (POD), ascorb c ac d o dase (AAO), po ypheno o dase (PPO), cata ase (CAT), gua aco pero dase (GPX), supero de d smutase (SOD), ascorbate pero dase (APX), and g utath one reductase (GR) (Noctor and Foyer, 1998; B o h na *et al*., 2003; G and Tuteja, 2010; Caverzan *et al*., 2012; So o *et al*., 2015). Among them, APX (EC 1.11.1.11) s an mportant enzyme or metabo sm o H_2O_2, and thus ncreases stab ty o membranes and prevents ce s rom njury (Yosh mura *et al*., 2000). Transgen c p ants nc ud ng *Nicotiana tabacum*, *Arabidopsis thaliana*, *Oryza sativa* *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 (CCAGGGTGGAAGGAATCTCA) 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 Bohp23. 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 uor de), and 20% (w/v) sorb to (Sh *et al.*, 2001). For each treatment, s p ants were used. The homogenate was centr uged at 13000 *g* and 4°C or 30 m n, and the supernatants were co ected or enzyme assays. The APX enzyme act v ty assay was per ormed n a react on m ture conta n ng 50 mM potass um phosphate (pH 7.0), 0.1 mM EDTA, 1 mM H_2O_2, 1.5 mM ascorbate, and 50 µ o the crude enzyme e tracts. The va ue o absorpt on at 290 nm was recorded at 80 s a ter the add t on o H_2O_2, and the concentrat on was ca cu ated accord ng to m cromo e o ascorbate o d zed per m nute (E = 2.8 mM/cm) (Na ano and Asada, 1981; L n *et al.*, 2010).

Electrical conductivity measurement. S ea d scs o 6 mm d ameter were punched out o each samp e and mmersed n test tubes w th 15 m d st ed water, and then were p aced n a 25°C water bath or 24 h. The n t a conduct v ty (EC_1) was measured us ng a DDS-11A conduct vty meter. The test tubes were then ept n a bo ng water bath or 30 m n, and coo ed to 25°C or a na conduct v ty determ nat on (EC_2). The re at ve EC va ue (%) was ca cuated as $EC_1 / EC_2 \times 100\%$ (Aposto ova *et al.*, 2008).

Statistical analysis. Compar sons between w d type and transgen c brocco p ants were per ormed us ng one-way ana ys s o var ance (ANOVA) and east s gn cant d erence (LSD) test (Tang and Zhang, 2013).

RESULTS

PCR detection of transgenic plants. To determ ne the b o og ca unct on o the *BoAPX* gene, *BoAPX* dr ven by CaMV 35S promoter was trans ormed nto brocco w d type p ants. A tota o seven over-e press on transgen c nes, name y *oe-apx07*, *oe-apx15*, *oe-apx22*, *oe-apx27*, *oe-apx32*, *oe-apx33* and *oe-apx49*, were screened out o 63 regenerated p ant ets by us ng anamyc n, and they were ater con rmed by PCR. Amp cat on products o the *npt*II gene were observed n both WT and transgen c p ants, however, the ragments o part a 35S promotor and *BoAPX* were present on y n those transgen c nes, and no band was observed n the WT ne (F g. 1).

Assessment of downy mildew resistance. Enough transgen c brocco p ants were generated us ng t ssue cu ture method. React on phenotypes were ass gned, and d sease nd ces were ca cu ated (Tab e 1). The contro p ants e h b ted a suscept b e react on w th d sease nde o 6.95, wh e the over-e press on nes showed d erent res stance c asses, rom LR to VR. Necrot c es ons, ch oros s, and heavy sporu at on were observed on eaves o w d type p ants wh ch was downy m dew suscept b e as we dent ed prev ous y. Necrot c es ons as we as sparse sporu at on were presented on the eaves o *oe-apx22* w th d sease nde o 5.10. *Oe-apx27* and *oe-apx49* were two

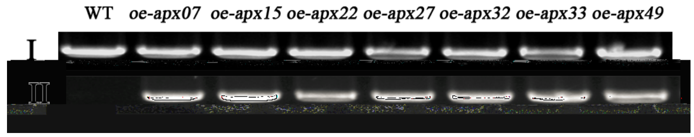

Fig. 1. PCR detect on o transgen c p ants. I: PCR detect on o *NPT*II gene n both WT and transgen c nes; II: PCR detect on o part a sequences o 35S promoter and *BoAPX* gene; WT: the contro p ant w th empty vector; *oe-apx07*, *oe-apx15*, *oe-apx22*, *oe-apx27*, *oe-apx32*, *oe-apx33* and *oe-apx49*: *BoAPX* over-e press on nes.

Table 1. Eva uat on o downy m dew nteract on–phenotype c asses o brocco transgen c nes.

L nes	Interact on–phenotype c ass[1] 0	1	3	5	7	9	P ant tested	D sease nde [2]	Res stance c ass[3]
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 E	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 necrot c ec s, no sporu at on; 1 = sma necrot c ec s, no sporu at on; 3 = necrot c ec s, one to ew sporang ophores; 5 = necrot c es ons, sparse scattered sporu at on usua y con ned to necrot c areas; 7 = necrot c es ons, somet mes w th accompany ng ch oros s, scattered, heavy to abundant sporu at on n both ch orot c and necrot c areas; 9 = necros s and some ch oros s may be ev dent, un orm y heavy sporu at on over aba a sur ace o ea .

[2] Va ues w th d erent owercase/uppercase etters are s gn cant y d erent at $P<0.05$ / $P<0.01$ accord ng to LSD's test, respect ve y.

[3] Res stance c asses based on d sease nd ces (DI) ca cu ated by W ams' ormu a: VR (very res stant), DI = 0-3.0; MR (moderate y res stant), DI = 3.1-5.0; LR (ow res stance), DI = 5.1-6.0; S (suscept b e), DI = 6.1-7.0; VS (very suscept b e), DI = 7.1-9.0.

moderate y res stant nes w th d sease nd ces o 3.40 and 4.75, respect ve y, and necrot c ec s and ew sporang ophores were observed on the r eaves. Interest ng y, our nes, *oe-apx07*, *oe-apx15*, *oe-apx32* and *oe-apx33*, showed a very h gh degree o res stance to downy m dew w th d sease nd ces o 2.94, 1.08, 1.79 and 2.94, respect ve y. No necrot c ec s or sporu at on was detected on eaves n *oe-apx15* (F g. 2).

Leaves o WT and seven over-e press on nes were used or APX enzyme act v ty assay. Compar ng w th the contro p ants, the APX enzyme act v ty were h gher n a the *BoAPX* over-e press on nes at 0 DAI, wh ch were probab y be due to *BoAPX* over-e press on. When cha enged w th *H. parasitica*, both the contro and over-e press on p ants e h b ted ncreased APX enzyme act v ty at 1 and 3 DAI, and decreased at 5 DAI. Compared w th the contro p ants, a the over-e press on nes showed h gher enzyme act v ty at 1 and 3 DAI. A ma ma eve was observed at 3 DAI n ne *oe-apx15* w th the va ue at 15.10 ± 0.23 U/g FW, wh ch was tw ce more than the contro at the same t me po nt (Tab e 2).

Assessment of electrical conductivity. The eaves o the contro and over-e press on p ants were sprayed w th *H. parasitica* and prepared or e ectr ca conduct v ty (REC) measurement. REC va ues were dent ed rom 0

Table 2. E ect o *Hyaloperonospora parasitica* on APX enzyme act v ty n eaves o transgen c brocco p ants (U/g FW).

Brocco nes	Days a ter nocu at on[1]			
	0 d	1 d	3 d	5 d
WT	5.13 ± 0.12 Cc	6.36 ± 0.35 Gg	6.63 ± 0.32 F	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 FG	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 va ues ± standard errors are shown (n = 6).
D erent owercase and uppercase etters nd cate s gn cant d erences among brocco nes at $p < 0.05$ and $p < 0.01$, respect ve y.

Table 3. The re at ve e ectr ca conduct v ty (%) change n eaves a ter spray o *Hyaloperonospora parasitica* n the over-e press on and contro p ants o brocco .

Brocco nes	Days a ter nocu at on[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 va ues ± standard errors are shown (n = 6).
D erent owercase and uppercase etters nd cate s gn cant d erences among brocco nes at $p < 0.05$ and $p < 0.01$, respect ve y.

Table 4. The assessment o APX enzyme act v ty n brocco eaves under heat stress (U/g FW).

Brocco nes	Days a ter 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 va ues ± standard errors are shown (n = 6).
D erent owercase and uppercase etters nd cate s gn cant d erences among brocco nes at $p < 0.05$ and $p < 0.01$, respect ve y.

Table 5. The re at ve e ectr ca conduct v ty (%) change n eaves under heat stress n the over-e press on and contro p ants o brocco .

Brocco nes	Days a ter 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 F	39.23 ± 0.61 E
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 va ± ± 0.87 E[illegible] Mean v- ±Mean v- ±Mean v ua T (.)-75m(EMC (ua T (.9.7 (5))-12 (e).)-14.6 (8)-5.9 (7)-125 (E)-22.

Env ronmenta stresses d srupt metabo c ba ance o ce s, resu t ng n enhanced product on o ROS. Deto - cat on o ROS needs more ant o dants. Over-e press on o *APX* genes ncreases APX enzyme act v ty and m- proves ROS scavenge ab ty. Over-e press on o *LetAPX* enhanced res stance to ch ng stress n tomato (*Lycopersicon esculentum*) (Duan *et al*., 2012). A *cAPX* gene rom pea (*Pisum sativum*) was nduced nto tomato. The trans ormed nes were proved to be ch ng and sa t stress to erant, and the APX act v ty n transgen c p ants was severa t mes h gher than that n the contro p ants (Wang *et al*., 2005). Over-e press on o a *Lycium chinense cAPX* gene n tobacco (*Nicotiana tabacum* cv. *SR-1*) showed h gher APX act v ty and enhanced sa t to erance (Wu *et al*., 2014). In our study, s gn cant h gher eve o APX enzyme act v ty was detected n over-e press on nes, and a the trans- ormed brocco p ants demonstrated ncreas ng d sease res stance and heat to erance. Whether under downy m dew nocu at on/heat stress or not, APX act v ty was much h gher n transgen c nes than that n WT p ants. In add t on, d erent enzyme act v ty n the over-e press ng p ants were observed when cha enged by downy m dew or h gh temperature, espec a y n *oe-apx07* and *oe-apx15* nes wh ch mp ed downy m dew and heat stress caused ROS accumu at on n p ants, and *BoAPX* over-e press on mproved ROS scaveng ng ab ty (Guan *et al*., 2015).

REC s regarded as an e ect ve nd cator or ce u ar membrane njury caused by o dat ve stress, dur ng e po- su 56 (e)eu(o)14. a30.5 (6)11.1 1pp e6vao-8.3 (R)15.m8-21.7 (n)a5a6-25.75 (1.1 (o-8.3 ( )17.6 ( )-39.5 ( )2)13 (t)-99.8 ( )81.1 (o-8.

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