THE CROP JOURNAL 3 (2015) 343–352

HOSTED BY

ELSEVIER

Available online at www.sciencedirect.com

ScienceDirect

The Crop Journal

Improvement of copper tolerance of *Arabidopsis* by transgenic expression of an allene oxide cyclase gene, *GhAOC1*, in upland cotton (*Gossypium hirsutum* L.)

Yuange Wang[a,1], *Huaihua Liu*[b,*,1], *Qingguo Xin*[c]

[a]*Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, China*
[b]*Postdoctoral Research Center of Shandong Shengfeng Seeds Co., Ltd., Jiaxiang 272400, China*
[c]*Yantai Academy of Agricultural Sciences, Yantai 265500, China*

ARTICLE INFO

Article history:
Received 17 April 2014
Received in revised form
15 February 2015
Accepted 16 February 2015
Available online 11 April 2015

Keywords:
Upland cotton
GhAOC
Expression pattern
Overexpression
Copper tolerance

ABSTRACT

Allene oxide cyclase (AOC, E 5.3.99.6) is an essential enzyme in the jasmonic acid (JA) biosynthetic pathway and mediates a wide range of adaptive responses. In this report, five *AOC* genes (*GhAOC1–GhAOC5*) were cloned from upland cotton (*Gossypium hirsutum* L.), sequenced, and characterized. Real-time PCR analysis indicated that the transcripts of *GhAOCs* were abundantly expressed in roots and less in fibers, and regulated in cotton plants under methyl jasmonate (MeJA) and $CuCl_2$ stresses. To investigate the role of *GhAOC* under copper stress, transgenic *Arabidopsis* plants overexpressing cotton *GhAOC1* under control of the *Cauliflower mosaic virus* 35S (CaMV 35S) promoter were generated. Compared to untransformed plants, *GhAOC1*-overexpressing *Arabidopsis thaliana* plants exhibited markedly higher survival rate, shoot fresh weight, shoot dry weight, and photosynthetic efficiency, and reduced cell membrane damage and lipid peroxidation under copper stress. This study provides the first evidence that *GhAOC1* plays an important role in copper stress tolerance.

1. Introduction

To adapt and survive, plants have developed complex defensive mechanisms to cope with a variety of abiotic and biotic stresses, such as pathogen attack, drought, water deficit, salinity, and heavy metals [1]. During plant adaptation to stress, JA, an important signaling chemical, may be generated from lipids. JA and its methyl ester, collectively referred to as jasmonates (JAs) [2], are important plant endogenous hormones involved in stress response [3,4].

The oxylipin pathway in plants is responsible for JA synthesis [5]. Under the catalysis of lipoxygenase, molecular oxygen is inserted into α-linolenic acid to produce (13S)-hydroperoxy-(9Z,11E,15Z)-octadecatrienoic acid. This product is dehydrated by a key enzyme, allene oxide synthase (AOS, EC 4.2.1.92) to an unstable allene oxide,

* *Corresponding author.*
E-mail address: liuhh@shofine.com (H. Liu).
Peer review under responsibility of Crop Science Society of China and Institute of Crop Science, CAAS.
[1] Yuange Wang and Huaihua Liu contributed equally to this work.

http://dx.doi.org/10.1016/j.cj.2015.02.004

which is cyclized by allene oxide cyclase (AOC) to 12-oxo-phytodienoic acid (OPDA). After by 12-oxo-phytodienoic acid reductase and several cycles of β-oxidation (+)-7-iso-JA is formed. AOC is considered especially important during JA biosynthesis, because its specificity leads to formation of the basic structure of JA through its ability to convert the unstable allene oxide derivative 12,13(S)-epoxyoctadecatrienoic acid (12,13-EOT) to OPDA [6,7].

AOC genes have been cloned from several plant species, including tomato [7], mangrove [8], *Arabidopsis thaliana* [9], barley [10], barrel medic [11], *Hyoscyamus niger* [12], *Camptotheca acuminate* [13], *Jatropha curcas* [14], *Physcomitrella patens* [15], *Glycine max* [6], and *Leymus mollis* [16]. Characterization of these genes from different plants has provided valuable information about their biochemical and physiological roles in adaptation to a variety of biotic and abiotic stresses. For example, overexpression of *JcAOC* in *Escherichia coli* conferred resistance to salt stress and low temperature [14]. Ectopic overexpression of an *AOC* gene cloned from *C. acuminate* using the viral 35S promoter improved tolerance against salt stress and low temperature in transgenic tobacco plants compared to wild-type controls [13]. *GmAOC5* transgenic tobaccos showed enhanced tolerance to oxidative stresses, while *GmAOC1*-expressing transgenic lines showed enhanced salinity stress tolerance [6]. However, the precise mechanisms of *AOC* function in these plant cell processes, particularly in regulating stress response, remain to be elucidated.

Plant *AOC* genes show specific and complex expression patterns in multiple organs. In soybean, *GmAOC2* showed higher expression only in roots, *GmAOC5* was expressed strongly only in stems, and *GmAOC3* was expressed highly in flowers [6]. In tomato, the *AOC* gene is expressed specifically in vascular bundles and the surrounding parenchymatic cells [7]. In contrast, four *AOC* genes in *A. thaliana* are constitutively expressed in all leaf tissues [9].

As one of the most important crops for the textile industry worldwide, cotton often suffers from various environmental stresses, such as drought, soil salinization, and heavy metals. Understanding the molecular basis of stress responses is accordingly a key target of cotton genetic improvement programs. To our knowledge, no reports in the literature have described the effect of *GhAOC* on abiotic tolerance in upland cotton. In the present study, we cloned five *AOC* genes, named as *GhAOC1*–*GhAOC5*, from cotton for the first time and analyzed their transcription profiles in various tissues and under different stress treatments. *GhAOC1* was overexpressed in *Arabidopsis* and the copper stress tolerance of the transgenic *Arabidopsis* plants was investigated.

2. Materials and methods

2.1. Plant materials, growth conditions, and treatments

Upland cotton (*Gossypium hirsutum* cv. Liaomian 9) seeds were surface-sterilized with 75% (*v*/*v*) ethanol for 1 min and 10% (*v*/*v*) H_2O_2 for 1 h, followed by washing with sterile distilled water. The sterilized seeds were germinated at 23 °C for 6 days and the germinated seedlings were transferred to plastic flats containing 13.5 L nutrient solution. The nutrient solution contained (mmol L^{-1}) KH_2PO_4, 0.2; $MgSO_4$, 0.5; KCl, 1.5; $CaCl_2$, 1.5; H_3BO_3, 1×10^{-3}; $(NH_4)_6Mo_7O_{24}$, 5×10^{-5}; $CuSO_4$, 5×10^{-4}; $ZnSO_4$, 1×10^{-3}; $MnSO_4$, 1×10^{-3}; Fe(III)-EDTA, 1×10^{-1}. The plants were grown in a controlled-environment growth chamber under the following conditions: 16 h light/8 h dark photoperiod with a light intensity of 350 μmol m^{-2} s^{-1}, 23 °C day and night temperatures, and relative humidity of 60–75%. Two-week-old seedlings with similar heights were transferred to nutrient solution containing 50 μmol L^{-1} MeJA and 120 μmol L^{-1} $CuCl_2$, respectively. At 0, 1, 3, 6, 12, 24, and 48 h after the stress treatments, the roots were quickly frozen in liquid nitrogen and stored at −70 °C for later RNA isolation.

Field grown cotton plants were used to analyze tissue-specific expression of *GhAOC*. After flowering, leaf, stem, root, petal, anther, stigma, ovule, and fiber tissues were sampled for gene expression analysis.

The wild-type (WT) *Arabidopsis* plants (Columbia ecotype) used for transformation were grown in a climate chamber at 22 °C, 70% relative humidity, and with a 16 h light/8 h dark photoperiod with a light intensity of 150 μmol m^{-2} s^{-1}.

2.2. RNA, DNA extraction, and cDNA synthesis

Total RNAs of cotton and *Arabidopsis* leaves were extracted with TRIzol reagent (Invitrogen, USA) and then treated with DNase I (Promega, USA). cDNAs were synthesized using oligo-$(dT)_{18}$ as anchor primer and M-MLV reverse transcriptase (Promega, USA) according to the manufacturer's instructions. Genomic DNA was extracted from cotton plants without stress using the cetyltrimethylammonium bromide method [17].

2.3. Isolation, sequencing, and analysis of GhAOCs

GhAOC genes were obtained by *in silico* cloning. Four *Arabidopsis AOC* genes (*AtAOC1*: NM_113475, *AtAOC2*: NM_113476, *AtAOC3*: NM_113477, *AtAOC4*: NM_101199) and six soybean *AOC* genes (*GmAOC1*: HM803106, *GmAOC2*: HM803107, *GmAOC3*: HM803108, *GmAOC4*: HM803109, *GmAOC5*: HM803110, *GmAOC6*: HM803111) were used as query probes for BLASTN searches of the public NCBI GenBank database. Based on the resulting sequence of interest, specific primers were designed to amplify the genomic and cDNA sequences of *GhAOC* in cultivar Liaomian 9 (Table 1). The polymerase chain reaction (PCR) was performed using *Pfu* DNA polymerase (Promega, Madison, WI, USA). The isolated genes were sequenced with an ABI3730 DNA Analyzer (Applied Biosystems Inc., Foster City, CA, USA).

2.4. Gene structure and bioinformatic analysis

Coding region was identified with ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The theoretical isoelectric point (pI) and molecular weight (Mw) of proteins were estimated with the Compute pI/Mw Tool (http://www.expasy.org/tools/pi_tool.html). The localization of proteins was predicted with PSORT (http://psort.nibb.ac.jp/).

Table 1 – Primer sequences used for *GhAOC* gene amplification.

Name	Sequence (5′–3′)	Name	Sequence (5′–3′)
GhAOC1-F	GGGTGGGCATCACGGCTGGACTC	*GhAOC1-R*	GAAGCTCAGCTGGCAAATCACC
GhAOC2-F	GGCCGCCTCGGGTAAATATAATTTC	*GhAOC2-R*	TAATTTATTTAATTAGTGAAATTTG
GhAOC3-F	ACTCTCAGTATGTCTGCTTTAAGAT	*GhAOC3-R*	TGACATTGCAAATGATTTAATTAG
GhAOC4-F	CTTACAACCCGATGGCCGCTTCA	*GhAOC4-R*	TAAATGATTTAATTAGTGAAATTT
GhAOC5-F	CACACTCTCAGTATGTCTGCTTTAA	*GhAOC5-R*	GCAAATGATTTAATTAGTGAAATT

2.5. Real-time PCR analysis

Real-time PCR was performed with SYBR Premix Ex *Taq*II (TaKaRa, Dalian, China) on a Master cycler ep realplex machine (Eppendorf, Hamburg, Germany). Triplicate quantitative assays were performed for each cDNA sample. *GhUbi* and *AtActin* were used as internal reference genes for assessing expression levels in cotton and *Arabidopsis*, respectively. The relative level of gene expression was calculated according to the method of Livak and Schmittgen [18]. Primer sequences for real-time PCR were listed in Table 2.

2.6. Construction of plant expression vectors and transformation of *Arabidopsis*

GhAOC was cloned in the pCAMBIA-1302 vector between *Bln* I and *Nco* I restriction sites under the control of the CaMV 35S promoter. The vector was transformed into *Agrobacterium* strain GV3101 by electroporation. The transgenic *Arabidopsis* plants were generated by the *Agrobacterium*-mediated floral dip method [19]. *Arabidopsis* plants were grown in a controlled culture room at 22 °C with relative humidity of 60–70% under long-day conditions (16 h light and 8 h dark).

2.7. Copper tolerance assay

For copper treatment, two T_3 homozygous plants (OX2 and OX6) and wild-type (WT) seeds were surface-sterilized and grown on MS medium for two weeks, then transferred into plastic pots filled with a 1:1 mixture of perlite and vermiculite and grown for five days before exposure to copper stress. To determine survival rate after copper treatment, three weeks old plants were exposed to copper stress by withholding water and irrigating with 120 μmol L^{-1} $CuCl_2$ for 10 days. After treatment, plants were transferred to the normal growth conditions described above. For measurement of survival rate, plants that had green and healthy young leaves were considered to have survived. Maximum photochemical efficiency (F_v/F_m), quantum yield of photosystem II (Φ_{PSII}), and malondialdehyde (MDA) concentration were determined as described previously [20,21]. Plant cell membrane damage rate (MDR) was measured with a microprocessor conductivity meter, DDS-12DW (Lida, Shanghai, China) according to the method of Sairam [22]. MDR (%) = initial electrical conductivity / electrical conductivity after boiling × 100. About 45 plants of each line were used in each stress experiment, and the experiments were performed at least three times.

2.8. Statistical analysis

ANOVA analysis of the experimental data was performed with SPSS 11.5 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Isolation and sequence analysis of *GhAOC* genes

Five *GhAOC* genes were identified and designated as *GhAOC1*–*GhAOC5*, respectively. They each contained a single open reading frame, encoding proteins of 253, 178, 246, 245, and 204 amino acids with molecular weights of 27.8, 19.8, 27.1, 27.0, and 22.4 kDa, respectively (Table 3). The similarities of the GhAOC proteins varied from 32.2% to 67.8% and their identities varied from 22.8% to 56.7%. All five proteins showed localization in the chloroplast by a PSORT analysis (http://psort.nibb.ac.jp/). Sequence alignment revealed that the GhAOC proteins shared high similarities in the C terminus,

Table 2 – Primer sequences used for real-time PCR.

Name	Sequence (5′–3′)	Name	Sequence (5′–3′)
AtActin-F	TGCTGACCGTATGAGCAAAG	*AtActin-R*	GATTGATCCTCCGATCCAGA
GhUbi-F	CTGAATCTTCGCTTTCACGTTATC	*GhUbi-R*	GGGATGCAAATCTTCGTGAAAAC
OPR3-F	GGTGCAGGGCGTTGAACGGAGTA	*OPR3-R*	GGTTGTGTAACGTGAAGGTAAGC
AtMYC2-F	TGGCAACCGTCGTATGATTTCT	*AtMYC2-R*	TCACCTCCTCATCAACAGCGTC
JAZ1-F	AGCTTCACTTCACCGGTTCTTGG	*JAZ1-R*	TCTTGTCTTGAAGCAACGTCGTC
PDF1.2-F	TGCTTCCATCATCACCCTTA	*PDF1.2-R*	TTCTGTGCTTCCACCATTGC
GhAOC1-F	CACCACCTCCCTCAAACCCATCCC	*GhAOC1-R*	GTATATGGCTTCATAGCGATCG
GhAOC2-F	GTATATGAGATGAATGAAAGAG	*GhAOC2-R*	GTGAAATTTGCAATGGCGGC
GhAOC3-F	CATTTCTTCTGGGATAATCAG	*GhAOC3-R*	GTTGCTAAAAGGGACAAGATC
GhAOC4-F	CCATTTCTTCTGGGATAATAAG	*GhAOC4-R*	CAAATCTCCCCTATATATTG
GhAOC5-F	GGGATAATCAGATCAACTTC	*GhAOC5-R*	GCTAAAAGGGACAAGATCAC

Table 3 – Overview of the five AOC genes identified in upland cotton.

Gene name	ORF length (bp)	Deduced polypeptide			Introns	PL[a]
		Length (AA)	Molecular weight (kDa)	Isoelectric point		
GhAOC1	762	253	27.8	8.95	1	Chlo
GhAOC2	537	178	19.8	5.85	2	Chlo
GhAOC3	741	246	27.1	8.51	2	Chlo
GhAOC4	738	245	27.0	9.06	2	Chlo
GhAOC5	615	204	22.4	9.21	3	Chlo

Chlo: chloroplast.
[a] Localization of GhAOC proteins determined by PSORT (http://psort.nibb.ac.jp/).

whereas the N-terminal region was divergent (Fig. 1). These divergent N-terminal regions resulted in variations in GhAOC protein length.

3.2. *Expression of GhAOCs in different tissues and under stress treatments*

To investigate their expression profiles in cotton tissues, expressions of the isolated five *GhAOC* genes in cotton were analyzed by real-time PCR. The relative transcript levels of the *GhAOC* genes in different tissues were shown in Fig. 2. The results showed that the five *GhAOC* genes were expressed mainly in cotton roots and at moderate to low levels in other tissues. The root-specific expression patterns of these *GhAOC* genes were probably associated with their functions in roots.

We next investigated the transcriptional responses of *GhAOCs* under MeJA and $CuCl_2$ treatments. Both treatments significantly increased the transcription of several *GhAOC*

```
GhAOC1    .MASTTSLKPIPSINLPSQSHRALASNFSYSKPFPFHGLNLSSVTETSSFTSSRSSNPFT     59
GhAOC2    ..........................................................      0
GhAOC3    ....MSALRSTISSGIIRSTS...PTHSLLPAASSFKPIKNPCLPQTHKLFTS.NSNTFS     52
GhAOC4    MAASSFALRSTISSGIISNPR...SKTLLPAANSSFKPIKSPSLTQTHKLFT......FS     51
GhAOC5    ....MSALRSTISSGIIRSTS...PTHSLLPAASSFKPIKNPCLPQTHKLFTS.NSNTFS     52
Consensus

GhAOC1    TTAFFFNKFKQEAAPHTPKPTKVQELHVYEMNERDRSSPAVLKLSQKPVNSLGDLVPFTN    119
GhAOC2    ................MAASDKVQELHVYEMNERDRGSPAYLRLSQKPVNSLGDIVPFSN     44
GhAOC3    APKRGFTCKSQAIPSDNSAPEKVQELHVYELNERDRGSPAYLRLSQKSVNSLGDLVPFSN    112
GhAOC4    APKRAFTCKSQAIPSDNSAPDKVQELHIYEMNERDRGSPAYLRLSQKSVNSLGDLVPFSN    111
GhAOC5    APKRGFTCKSQAIPSDNSAPEKVQELHVYELNERDRGSPAYLRLSQKSVNSLGDLVPFSN    112
Consensus                       kvqelh ye nerdr spa l lsqk vnslgd vpf n

GhAOC1    KLYSGDLQKRVGITAGLCVLIQHVPEKKGDRYEAIYSFYFGDYGHLSVQGPYLTYEDTYL    179
GhAOC2    KIYRGDLEKRIGITAGMCILIEHKPELKGDRYEAIFSFYFGDYGHIAVQGPYLTYQDSYL    104
GhAOC3    KIYRGDLEKRIGITSGICILIEHKPEMKGDRYEAIFSFYFGDYGHIAVQGPYLTYQDTYL    172
GhAOC4    KIYRGDLEKRIGITAGICILIEHKPEMKGDRYEAIFSFYFGDYGHIAVQGPYLTYQDTFL    171
GhAOC5    KIYRGDLEKRIGITSGICILIEHKPEMKG.............................    141
Consensusk y gdl kr git g c li h pe kg

GhAOC1    AVTGGSGIFEGAYGQVKLHQIVFPMKLYYTFYLKGIGDLPAELLGKPVPPSPAVEPSAAA    239
GhAOC2    AITGGSGIFEGVSGQVKLHQIVFPFKIFYTFYLKGIGELPEELLCKPVDPHPAVEAAPAA    164
GhAOC3    AITGGSGIFEGVSGQVKLHQIVFPFKIFYTFYLKGIGELPEELLCKPVDPHPAVEAVPAA    232
GhAOC4    AIKGGSGIFEGVTGQVKLRQIVFPFKIFYTFYLKGIGELPEELLCKPVEPHPAVEAFPAA    231
GhAOC5    ...........VSGQVKLHQIVFPFKIFYTFYLKGIGELPEELLCKPVDPHPAVEAVPAA    190
Consensus             gqvkl qivfp k  ytfylkgig lp ell kpv p pave   aa

GhAOC1    KATEPHGSIPNFTN                                                  253
GhAOC2    KACEPHAAIANFTN                                                  178
GhAOC3    KACEPHATIANFTN                                                  246
GhAOC4    KACEPHAAIANFTN                                                  245
GhAOC5    KACEPHATIANFTN                                                  204
Consensuska eph  i nftn
```

Fig. 1 – Alignment of GhAOC deduced amino acid sequences. Identical amino acids were shaded in black.

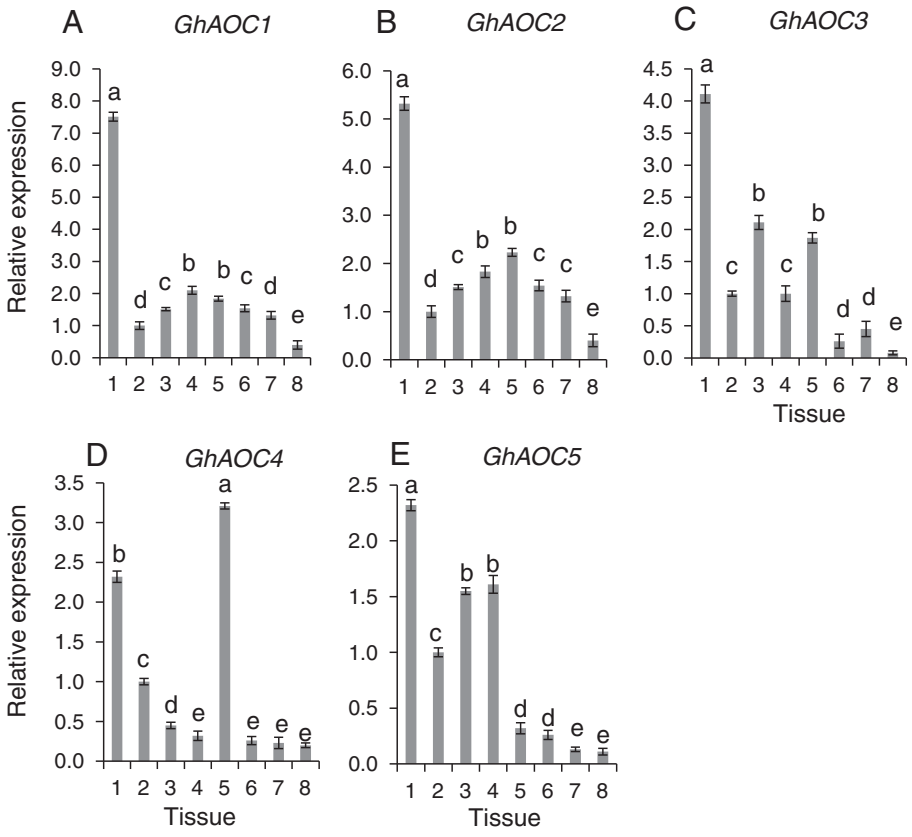

Fig. 2 – Real-time PCR expression profiles of *GhAOC* genes in different tissues. Different letters (A to E) indicated statistical significance at the 0.05 probability level among different cotton tissues. Error bars represented standard deviation (SD) of three biological replicates, each was analyzed with three technical replicates. 1: roots; 2: hypocotyls; 3: cotyledons; 4: leaves; 5: anthers; 6: petals; 7: ovules; 8: fibers.

genes compared to the control (0 h). However, this upregulation was time-dependent, and the expression of different genes peaked at different times after exposure to these stresses. For example, under MeJA treatment, all *GhAOC* genes were upregulated and peaked at different times, with *GhAOC2*, *GhAOC4*, and *GhAOC5* reaching the highest expression level in the minimum time (Fig. 3). In the copper stress treatment, *GhAOC1* was significantly upregulated by 120 μmol L^{-1} $CuCl_2$, whereas *GhAOC5* showed no obvious change (Fig. 4). Collectively, our results indicated a divergence of expression profiles and levels in these five *GhAOC* genes.

3.3. *GhAOC1* expression conferred copper tolerance in *Arabidopsis* plants

Because the expression of *GhAOC1* was markedly upregulated by $CuCl_2$, we evaluated the copper tolerance of *GhAOC1*-overexpressing plants under $CuCl_2$ stress. Under normal growth conditions, no significant difference was observed in either WT or transgenic seedlings. The positive *GhAOC1* transgenic plants were confirmed by RT-PCR (Fig. 5-A). After copper treatment, most WT plants were chlorotic and wilting (Fig. 5-B), and the survival rate of the WT plants was only 35.5%, whereas the *GhAOC1*-overexpressing plants remained green and survived (Fig. 5-C). Compared with the control, copper stress greatly increased JA content, and this increase was much greater in the transgenic than in the WT plants (Fig. 5-D). Under control conditions, there was no significant difference in shoot fresh and dry weights between WT and transgenic plants. After the plants were subjected to copper stress for 10 days, shoot fresh and dry weights were reduced in the WT and transgenic plants, indicating that copper stress impeded plant growth and development. High $CuCl_2$ treatment reduced shoot fresh weight and dry weight more severely in WT than in transgenic plants (Fig. 5-E, F), implying that overexpressing *GhAOC1* increased the tolerance of the plants to copper stress.

3.4. *GhAOC1* expression led to a reduction in MDA and MDR and improved photosynthetic activity under copper stress in transgenic *Arabidopsis* plants

To investigate the physiological mechanisms underlying the involvement of *GhAOC1* in cotton tolerance to copper stress, F_v/F_m, Φ_{PSII}, MDA concentration, and MDR were determined in the youngest expanded leaf before and after copper treatment. The values of F_v/F_m, Φ_{PSII}, MDA, and MDR showed no significant differences between the control and transgenic plants without treatment. Following exposure to copper stress for 10 days, the values of F_v/F_m and Φ_{PSII} were significantly reduced. This reduction was much greater in control than in transgenic plants (Fig. 6-A, B). Copper stress greatly increased

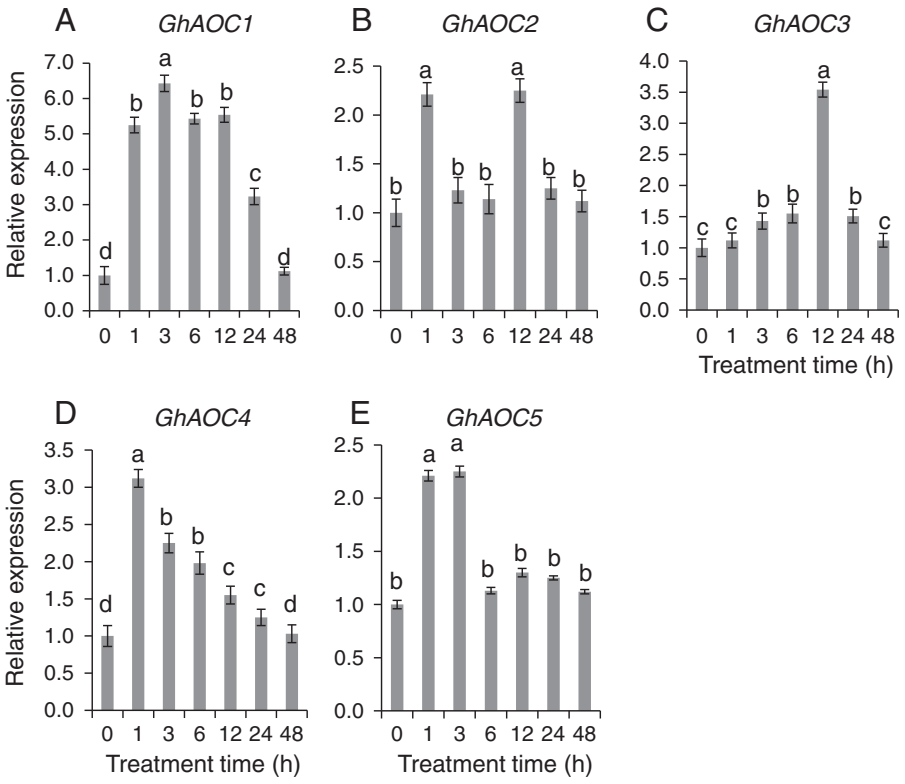

Fig. 3 – The expression patterns of *GhAOC* genes in response to MeJA. Each value is the mean ± SD of at least three independent measurements. Different letters indicated a significant difference at the 0.05 probability level.

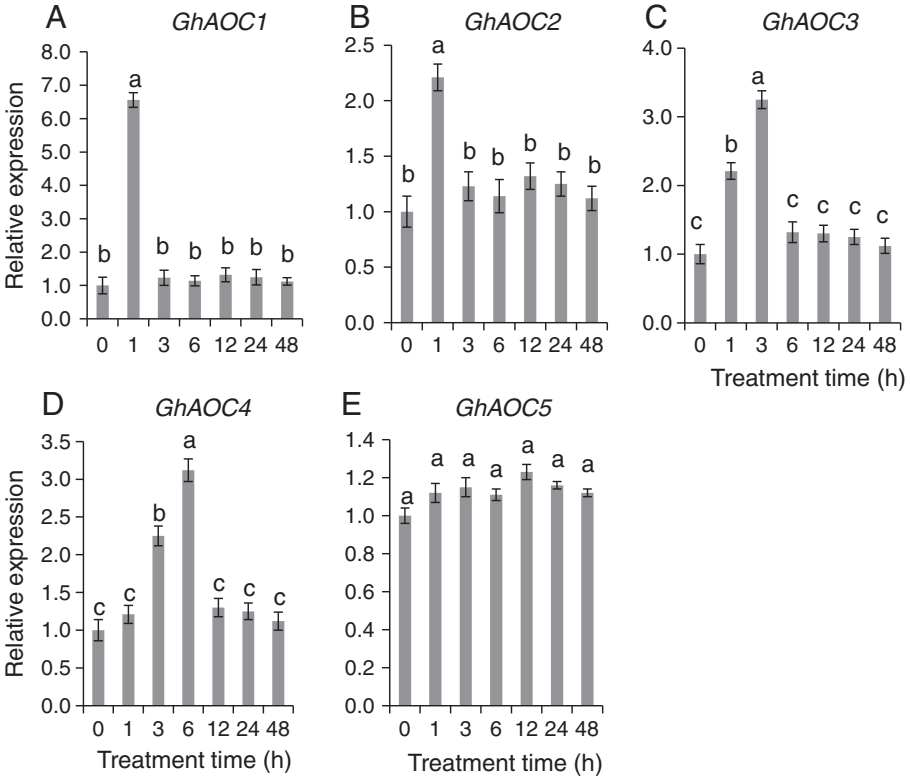

Fig. 4 – Expression patterns of *GhAOC* genes in response to $CuCl_2$. Each value was the mean ± SD of at least three independent measurements. Different letters on the same column indicated a significant difference at the 0.05 probability level.

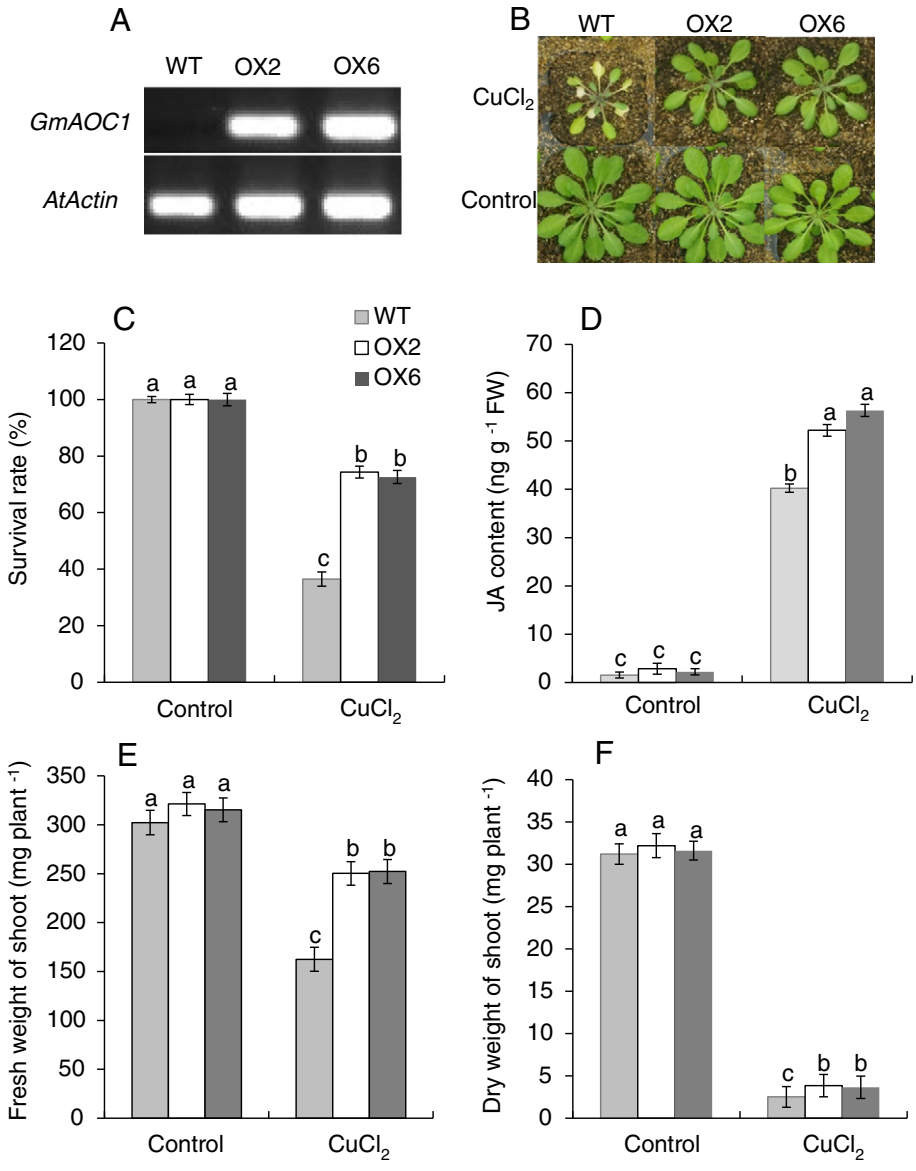

Fig. 5 – Stress response of *GhAOC1*-expressing *Arabidopsis* seedlings. (A) Growth of WT and transgenic lines without or with $CuCl_2$ stress. (B) Expression of *GhAOC1* in different transgenic *Arabidopsis* lines. (C) Survival rates of WT and *GhAOC1*-overexpressing seedlings after copper treatment. (D) JA content of WT and *GhAOC1* transgenic plants under copper treatment. (E) Fresh weight of WT and *GhAOC1*-overexpressing seedlings after copper treatment. (F) Dry weight of WT and *GhAOC1*-overexpressing seedlings after copper treatment. Different letters indicated a significant difference at the 0.05 probability level.

MDA concentration and MDR compared to the normal condition, and this increase was much greater in the WT than in the transgenic plants (Fig. 6-C, D).

3.5. *Copper-induced expression of GhAOC1in Arabidopsis plants upregulated the JA pathway*

The effect of overexpressing *GhAOC1* in *Arabidopsis* on the transcription of genes in the JA pathway was investigated by monitoring the accumulation of *OPR3* (a JA synthesis enzyme downstream of AOC), *Jasmonate-ZIM-Domain Protein1* (*JAZ1*) and *AtMYC2* (JA signaling genes), and *Plant Defensin1.2* (*PDF1.2*, a JA signaling pathway gene). The expression of these four genes was increased in transgenic compared with WT plants under copper treatment (Fig. 7).

4. Discussion

Previous studies showed that the *AOC* genes belong to a multigene family. There are four homologous *AOC* genes in *Arabidopsis* and six in soybean [6,9]. Tetraploid upland cotton may contain several *AOC* genes in its large genome, but to date little systematic identification of full-length *AOC* genes in

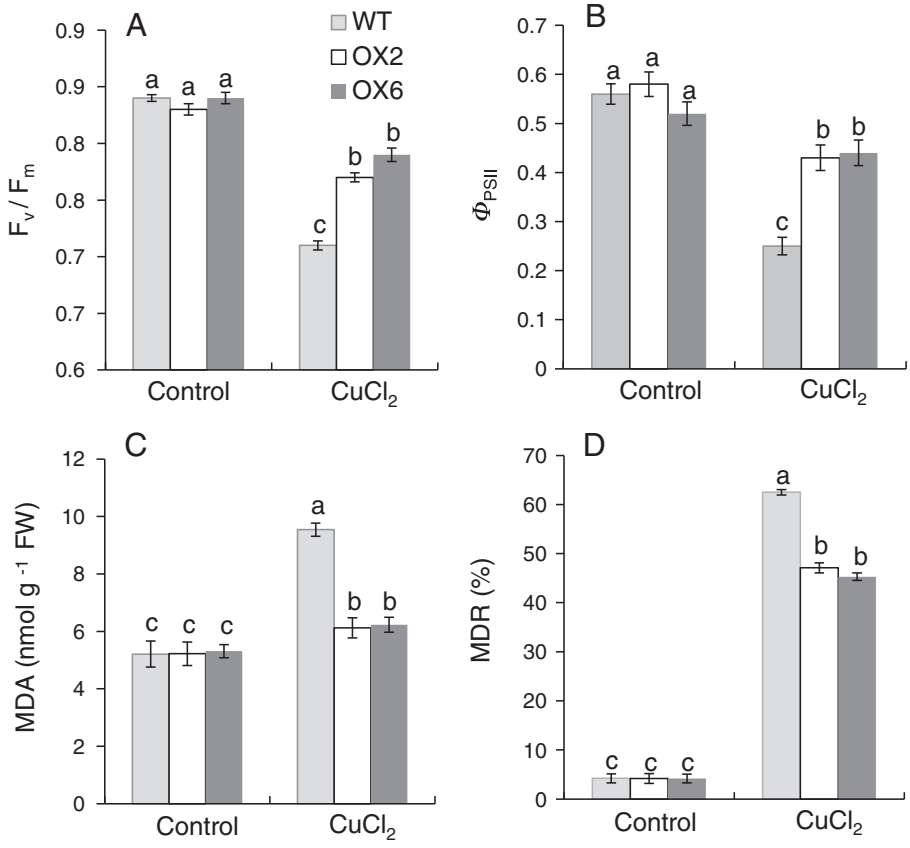

Fig. 6 – F_v/F_m, Φ_{PSII}, MDA concentration, and MDR in plants. (A) Changes in F_v/F_m with or without copper stress treatment. (B) Values of Φ_{PSII} before and after 120 μmol L^{-1} $CuCl_2$ treatment. (C) MDA concentration in transgenic and WT plants after 10 day treatment with 120 μmol L^{-1} $CuCl_2$. (D) MDR of transgenic and WT seedlings treated with 120 μmol L^{-1} $CuCl_2$ for 10 days. Different letters indicated a significant difference at the 0.05 probability level.

this important economic crop has been reported. In the present study, five *GhAOC* genes were identified in cotton. All five *GhAOC* proteins are conserved in their C termini and divergent in their N-terminal regions, like other plant AOC proteins [15]. Analysis of tissue-specific expression showed that *GhAOCs* were abundantly expressed in roots (Fig. 3). The tissue expression profile of *GhAOC* was in agreement with results in soybean, in which *GmAOC2* was expressed predominantly in roots [6]. This finding seems reasonable, given that roots are buried in soil and may sense the presence of copper ions.

The dynamic expression patterns of *GhAOC* were affected by MeJA and $CuCl_2$ (Fig. 4), suggesting that *GhAOCs* are involved in the crosstalk of the environmental stress response in cotton. MeJA is an important cellular regulator responding to various environmental conditions in plants [3]. The observation that *GhAOCs* were upregulated by MeJA (Fig. 4-A) suggests that the induction of *GhAOC* gene by $CuCl_2$-induced stress is associated with the MeJA-mediated signaling pathway. Further work is planned to clarify the molecular mechanism through which the GhAOC proteins interact with the signal transduction system of MeJA.

Plants respond to environmental stress conditions by adjusting various biochemical and physiological processes [23]. The biochemical and physiological basis of tolerance of *GhAOC1* transgenic plants was analyzed by studying changes in photosynthetic efficiency, MDR, and MDA concentration under copper stress. After the plants were treated with copper stress for 10 days, survival rates and shoot fresh and dry weights were significantly higher in transgenic lines than in the WT (Fig. 5), implying that *GhAOC1* conferred tolerance to copper stress. Chlorophyll fluorescence in intact leaves is a reliable, noninvasive method for monitoring photosynthetic changes and reflects the physiological conditions of plants. F_v/F_m is usually used as an important parameter to assess the photooxidative damage to photosystem II (PSII) and decrease in its value indicates damage to PSII [24]. Efficiency of PSII (Φ) [25] measures the amount of absorbed light by chlorophyll molecules associated with PSII system and is described as effective quantum yield (Φ_{PSII}). Imposition of copper stress resulted in a marked reduction in the F_v/F_m and Φ_{PSII} values of the transgenic plants, and this reduction was greater in the WT plants (Fig. 6-A, B). These observations indicated that overexpression of *GhAOC1* resulted in enhanced stability of PSII under copper stress. Maintenance of membrane stability is a major component of environmental stress tolerance [26]. The MDR under stress conditions can be estimated by measurement of electrolyte leakage from plant cells. MDA,

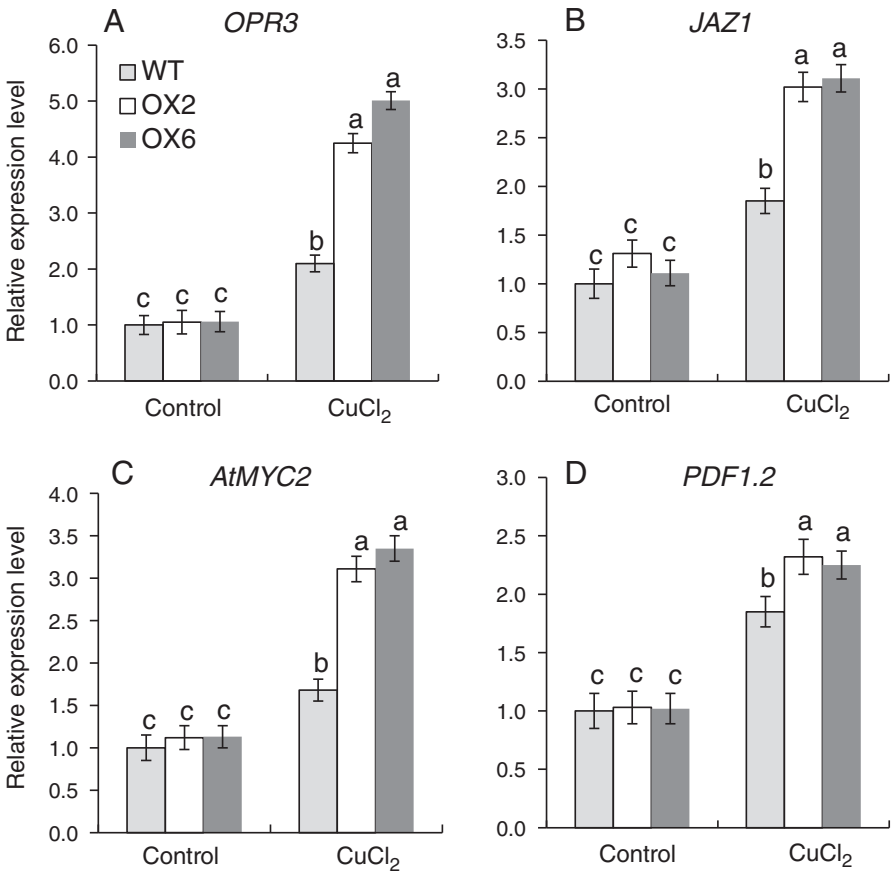

Fig. 7 – Expression levels of *OPR3* (A), *JAZ1* (B), *AtMYC2* (C), and *PDF1.2* (D) in WT and transgenic plants with or without copper stress. Each value was the mean ± SD of at least three independent measurements. Different letters indicated a significant difference at the 0.05 probability level.

produced as a result of peroxidation of membrane lipids, is used as a marker of stress-induced oxidative damage at the cellular level [27]. The results obtained in the present study showed that copper stress for 10 days significantly reduced MDA and MDR in transgenic plants relative to that in WT plants (Fig. 6-C, D), clearly showing that lipid peroxidation and cell membrane damage reduction in transgenic plants were caused by overexpression of *GhAOC*.

To investigate the enhanced copper tolerance at the molecular level, expression levels of several JA signaling genes were measured in transgenic and WT plants with or without copper stress. *GhAOC1*-overexpressing transgenic *Arabidopsis* lines displayed increased expression levels of *OPR3*, *JAZ1*, *AtMYC2*, and *PDF1.2* under copper stress (Fig. 7). Our results suggested that the higher tolerance to copper stresses observed in *GhAOC1*-overexpressing *Arabidopsis* was the result of the increased expression levels of these genes.

In summary, this study identified and characterized five *GhAOC* genes from upland cotton. *GhAOCs* were expressed mainly in roots and significantly upregulated by MeJA and $CuCl_2$ treatments. Overexpression of *GhAOC1* led to increased expression of several JA signaling genes and significantly higher survival rate, JA accumulation, shoot fresh and dry weight, and photosynthetic efficiency under copper stress. *GhAOC1* may prove to be a useful gene for molecular breeding of important crops to improve copper stress tolerance.

Acknowledgments

This study was supported by the project of Modern Seed Industry Enterprise Science and Technology Development of Shandong Province, China (SDKJ2012QF003).

REFERENCES

[1] E.E. Farmer, R.R. Johnson, C.A. Ryan, Regulation of expression of proteinase inhibitor genes by methyl jasmonate and jasmonic acid, Plant Physiol. 98 (1992) 995–1002.
[2] C. Wasternack, B. Hause, Jasmonates and octadecanoids: signals in plant stress responses and plant development, Prog. Nucleic Acid Res. 72 (2002) 165–221.
[3] R.A. Creelman, J.E. Mullet, Biosynthesis and action of jasmonates in plants, Annu. Rev. Plant Physiol. Plant Mol. Biol. 48 (1997) 355–381.
[4] S. Parchmann, H. Gundlach, M.J. Mueller, Induction of 12-oxo-phytodienoic acid in wounded plants and elicited plant cell cultures, Plant Physiol. 115 (1997) 1057–1064.
[5] M. Stumpe, I. Feussner, Formation of oxylipins by CYP74 enzymes, Phytochem. Rev. 3 (2006) 347–357.
[6] Q. Wu, J. Wu, H. Sun, D. Zhang, D. Yu, Sequence and expression divergence of the *AOC* gene family in soybean: insights into functional diversity for stress responses, Biotechnol. Lett. 33 (2011) 1351–1359.

[7] J. Ziegler, I. Stenzel, B. Hause, H. Maucher, M. Hamberg, R. Grimm, M. Ganal, C. Wasternack, Molecular cloning of allene oxide cyclase: the enzyme establishing the stereochemistry of octadecanoids and jasmonates, J. Biol. Chem. 275 (2000) 19132–19138.
[8] A. Yamada, T. Saitoh, T. Mimura, Y. Ozeki, Expression of mangrove allene oxide cyclase enhances salt tolerance in *Escherichia coli*, yeast, and tobacco cells, Plant Cell Physiol. 43 (2002) 903–910.
[9] I. Stenzel, B. Hause, O. Miersch, T. Kurz, H. Maucher, H. Weichert, J. Ziegler, I. Feussner, C. Wasternack, Jasmonate biosynthesis and the allene oxide cyclase family of *Arabidopsis thaliana*, Plant Mol. Biol. 51 (2003) 895–911.
[10] H. Maucher, I. Stenzel, O. Miersch, N. Stein, M. Prasad, U. Zierold, P. Schweizer, C. Dorer, B. Hause, C. Wasternack, The allene oxide cyclase of barley (*Hordeum vulgare* L.): cloning and organ-specific expression, Phytochemistry 65 (2004) 801–811.
[11] S. Isayenkov, C. Mrosk, I. Stenzel, D. Strack, B. Hause, Suppression of allene oxide cyclase in hairy roots of *Medicago truncatula* reduces jasmonate levels and the degree of mycorrhization with *Glomus intraradices*, Plant Physiol. 139 (2005) 1401–1410.
[12] K.J. Jiang, Z.H. Liao, Y. Pi, Z.S. Huang, R. Hou, Y. Cao, Q. Wang, X.F. Sun, K.X. Tang, Molecular cloning and expression profile of a jasmonate biosynthetic pathway gene for allene oxide cyclase from *Hyoscyamus niger*, Mol. Biol. 42 (2008) 381–390.
[13] Y. Pi, K.J. Jiang, Y. Cao, Q. Wang, Z. Huang, L. Li, L. Hu, W. Li, X. Sun, K. Tang, Allene oxide cyclase from *Camptotheca acuminata* improves tolerance against low temperature and salt stress in tobacco and bacteria, Mol. Biotechnol. 41 (2009) 115–122.
[14] B. Liu, W. Wang, J. Gao, F. Chen, S. Wang, Y. Xu, L. Tang, Y. Jia, Molecular cloning and characterization of a jasmonate biosynthetic pathway gene for allene oxide cyclase from *Jatropha curcas*, Acta Physiol. Plant. 32 (2010) 531–539.
[15] T. Hashimoto, K. Takahashi, M. Sato, P.K.G.S.S. Bandara, K. Nabeta, Cloning and characterization of an allene oxide cyclase, PpAOC3, in *Physcomitrella patens*, Plant Growth Regul. 65 (2011) 239–245.
[16] M.E. EltayebHabora, A.E. Eltayeb, M. Oka, H. Tsujimoto, K. Tanaka, Cloning of allene oxide cyclase gene from *Leymus mollis* and analysis of its expression in wheat-*Leymus* chromosome addition lines, Breed. Sci. 63 (2013) 68–76.
[17] M.G. Murray, W.F. Thompson, Rapid isolation of high molecular weight plant DNA, Nucleic Acids Res. 8 (1980) 4321–4326.
[18] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method, Methods 25 (2001) 402–408.
[19] S.J. Clough, A.F. Bent, Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*, Plant J. 16 (1998) 735–743.
[20] S. Lal, V. Gulyani, P. Khurana, Overexpression of *HVA1* gene from barley generates tolerance to salinity and water stress in transgenic mulberry (*Morus indica*), Transgenic Res. 17 (2008) 651–663.
[21] R.S. Dhindsa, P. Plumb-Dhindsa, T.A. Thorpe, Leaf senescence correlated with increased levels of membrane permeability and lipid peroxidation, and decreased levels of superoxide dismutase and catalase, J. Exp. Bot. 32 (1981) 93–101.
[22] R.K. Sairam, Effect of moisture stress on physiological activities of two contrasting wheat genotypes, Indian J. Exp. Biol. 32 (1994) 594–597.
[23] B. Singh, H. Chauhan, J.P. Khurana, P. Khurana, P. Singh, Evidence for the role of wheat eukaryotic translation initiation factor 3 subunit g (*TaeIF3g*) in abiotic stress tolerance, Gene 532 (2013) 177–185.
[24] K. Maxwell, G.N. Johnson, Chlorophyll fluorescence—a practical guide, J. Exp. Bot. 51 (2000) 659–668.
[25] J. Harbinson, B. Genty, N.R. Baker, Relationship between the quantum efficiencies of photosystems I and II in pea leaves, Plant Physiol. 90 (1989) 1029–1034.
[26] J. Levitt, Responses of plants to environmental stresses: water, radiation, salt and other stresses, Academic Press, New York, 1980.
[27] M. Jain, G. Mathur, S. Koul, N.B. Sarin, Ameliorative effects of proline on salt stress induced lipid peroxidation in cell lines of groundnut (*Arachis hypogaea* L.), Plant Cell Rep. 20 (2001) 463–468.