Genetics and Molecular Research

Expression profile analysis of ascorbic acid-related genes in response to temperature stress in the tea plant, *Camellia sinensis* (L.) O. Kuntze

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ABSTRACT. Ascorbic acid (AsA), also known as ascorbate or vitamin C, is a natural organic compound in green plants that has antioxidant properties, and is an essential nutrient for humans. The tea plant, *Camellia sinensis* (L.) O. Kuntze, is an important global economic crop. Here, the expression profiles of genes related to AsA biosynthesis and recycling were analyzed in tea plants in response to temperature stress. Eighteen genes involved in AsA biosynthesis and recycling pathways were identified based on the transcriptome database. The expression levels of *CsPGI1* in two varieties of tea plants ('Yingshuang' and 'Huangjinya') increased, peaked at 4 h, and then decreased in response

to cold stress. In 'Yingshuang', the genes involved in AsA biosynthesis pathway rapidly responded to heat stress and substantially increased their expression levels at 1 h. The expression levels of *CsMDHAR*, *CsDHAR1*, and *CsDHAR2* increased sharply at 1 h in response to heat stress in 'Yingshuang'. In contrast, the expression levels of *CsMDHAR*, *CsDHAR1*, and *CsDHAR2* in 'Huangjinya' gradually increased during heat treatment from 1 to 24 h. The expression trends of two *DHAR* isoforms differed in 'Huangjinya' during cold stress. The expression patterns of AsA-related genes differed in the different tea plant varieties and depended on temperature. The genes involved in AsA biosynthesis and recycling pathways were induced by heat and cold stress. Our study provides useful data with which to improve the resistance of tea plants to cold and heat stress.

Key words: Ascorbic acid; Biosynthetic synthesis; *Camellia sinensis*; Expression profile; Recycling pathway; Temperature stress

INTRODUCTION

The tea plant, *Camellia sinensis* (L.) O. Kuntze, is an important economic crop that is mainly grown in Southeast Asia, and has been cultivated for nearly 2000 years in China (Banerjee, 1992; Chen et al., 2009). Tea is made using the leaves of the tea plant. Humans could absorb minerals, several essential amino acids, and vitamins from tea (Graham, 1992; Aucamp et al., 2000). The quality and yield of tea plants are largely influenced by environmental temperature (Tanton, 1982). Abiotic stress (e.g., temperature stress) affects tea plant growth, and may affect the expression levels of genes related to ascorbic acid (AsA). Temperature stress-related damage instigates a series of physiological changes. Oxidative stress, which is caused by the accumulation of reactive oxygen species (ROS), is thought to be the main reason for temperature stress-related damage in plants (Baek and Skinner, 2003; Xu et al., 2006). As an antioxidant, AsA plays an important role in scavenging excess ROS under abiotic stress (Noctor and Foyer, 1998).

AsA is also known as ascorbate or vitamin C, and is widely recognized as being one of the most abundant antioxidants, and for its vital roles in plant-related processes, such as hormone biosynthesis, defense mechanisms, photosynthesis, florescence regulation, cell division, growth regulation, and senescence (Smirnoff, 2011). Four AsA biosynthetic pathways exist in plants: L-gal (L-galactose), L-gulose, D-galacturonate, and *myo*-inositol (Wheeler et al., 1998; Agius et al., 2003; Wolucka and Van Montagu, 2003; Lorence et al., 2004). The L-galactose pathway has been investigated thoroughly, and is considered a major AsA biosynthesis pathway in several plant species (Wang et al., 2015). Previous studies have investigated the expression profiles of AsA-related genes in response to temperature stress conditions in apples and tomatoes (Ma et al., 2008; Ioannidi et al., 2009). To our knowledge, no studies have evaluated them in the tea plant till now.

Twelve genes that are involved in the AsA biosynthesis pathway (*CsPMM*, *CsGGP*, *CsGalDH*, *CsPGI1*, *CsPGI2*, *CsPMI*, *CsGalLDH*, *CsGPP*, *CsGMP*, *CsGME*, *CsMIOX*, and *CsGalUR*) and six that are related to the AsA recycling pathway (*CsMDHAR*, *CsDHAR1*, *CsDHAR2*, *CsAO*, *CsGR*, and *CsAPX*) were identified and selected from tea plants based on the transcriptome database (Wu et al., 2014). These 18 genes play important roles in AsA

biosynthesis and metabolism in higher plants (Wang et al., 2015). In the present study, the expression levels of these genes were assessed and compared in response to high- or low-temperature stress in two tea plant varieties, 'Yingshuang' and 'Huangjinya', both of which are suitable for processing into green tea and are cultivated in an inland, subtropical monsoon climate. Our objective was to identify genes that are involved in AsA biosynthesis and recycling pathways and temperature stress resistance. Our results could facilitate studies into the adaptation of tea plants to temperature stress.

MATERIAL AND METHODS

Plant materials and growth conditions

Four-year-old cut seedlings of *C. sinensis* cultivars 'Yingshuang' and 'Huangjinya' were used. They were cultivated in a growth chamber with acidic soil at the State Key Laboratory of Crop Genetics and Germplasm Enhancement, Nanjing Agriculture University (Figure 1). The growth conditions were 25°C and 70 ± 10% relative humidity. Leaf samples were obtained at 0, 1, 2, 4, 8, 12, and 24 h under heat treatment (38°C) and cold treatment (4°C). Three young leaves from each of three plants of each variety were removed as samples, frozen in liquid nitrogen, and then stored at -80°C for RNA extraction.

Figure 1. Two tea plant cultivars: 'Yingshuang' and 'Huangjinya'.

RNA isolation and cDNA reverse transcription (RT)

The total RNA of the leaves was isolated using a RNA extraction kit (Huayueyang Biotech Co., Ltd., Beijing, China). The RNA concentration was measured using a NanoDrop™ 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). cDNA was synthesized using a PrimeScript™ RT reagent kit (TaKaRa, Dalian, China), in accordance with the manufacturer protocols.

Selection of genes related to AsA biosynthesis and recycling pathways in tea plants

Twelve genes that encode enzymes involved in the AsA biosynthetic pathway were selected in the tea plant transcriptome database (Wu et al., 2014): two *PGI* isoforms (phosphoglucose isomerase), *PMI* (phosphomannose isomerase), *PMM* (phosphomannose

mutase), *GMP* (GDP-D-mannose pyrophosphorylase), *GME* (GDP-D-mannose-3',5'-epimerase), *GGP* (GDP-L-galactose phosphorylase), *GPP* (L-galactose-1-P phosphatase), *GalDH* (L-galactose dehydrogenase), *GalLDH* (L-galactono-1,4-lactone dehydrogenase), *GalUR* (D-galacturonate reductase), and *MIOX* (*myo*-inositol oxygenase). Six additional genes that encode enzymes related to AsA recycling and degradation pathways were also selected in the tea plant transcriptome database (Wu et al., 2014): *AO* (ascorbate oxidase), *APX* (ascorbate peroxidase), two *DHAR* isoforms (dehydroascorbate reductase), *GR* (glutathione reductase), and *MDHAR* (monodehydroascorbate reductase).

RT-quantitative polymerase chain reaction (qPCR) analysis

The expression levels of twelve genes related to AsA biosynthesis pathways and six related to AsA recycling and degradation pathways were detected by RT-qPCR method. The specific primers of these genes for RT-qPCR were designed using Primer Premier 6 (Table 1). RT-qPCR was conducted in an Applied Biosystems IQ5 Real-Time PCR System (Applied Biosystems, USA). The reaction program was set as follows: 95°C for 30 s; followed by 40 cycles at 95°C for 5 s and 55°C for 25 s. The reaction volume was 20 μL, which contained 2 μL diluted cDNA strand, 7.2 μL ddH_2O, 10 μL SYBR® Premix *Ex Taq* (TaKaRa, Dalian, China), and 0.4 μL each primer. *Csactin* was used to normalize the expression levels of the AsA-related genes. Relative gene expression was calculated based on a previously described method (Pfaffl, 2001).

Table 1. Primer sequences of genes related to ascorbic acid biosynthesis and recycling pathways in tea plants for reverse transcription-quantitative real time polymerase chain reaction.

Name	Forward primer (5'-3')	Reverse primer (5'-3')
CsPMM	CCACATTATTAGCTTCCTTCTCGTCAC	CCAACAACACCAACTGTAACAACCTT
CsGGP	ATCTTCCTTGTACCACAGTGTTATGCT	TGCCTCCTCGTAGTCCTTCTTCC
CsGME	AACTACGGAGCATACACCTATGAGAAC	CTAGCAATGTGCGAGGCAATGAATC
CsGMP	GAACTCGGTTGAGACCATTGACACTT	CCACTTCACTCACTCCAATAGCCTTG
CsGPP	GCTGCTGGTGCTGTGGTAGAAT	CTAGAAGTGACTGCTCCACCTTATCG
CsGalLDH	GGCGGCATTGTTCAGGTTGGT	GTCCACAGCGAGCAAGATAGAATAGTT
CsGalDH	GAGAGTGACTAGGAGCATTGATGAGAG	CCAAGCGGAAGTCCTGTAATACCAA
CsPMI	TCTGCGGTCAATATTCACTCAACTCAT	TGTTCCTTATCTGTCAACTGCCTCAC
CsPGI1	CATTGTGAAGAGTCAGCAACCTGTGTA	CGATTGCCAGAGAAGGTCTTGTGAG
CsPGI2	CGATGTCGTCAGTGGTAAGATTAAGC	TTATCTTGAGAGGCGGATTATCAGGAG
CsAPX	AGCAAGGTCACGAAGCCAACAAT	GCAACAACTCCAGCCAACTGATAGA
CsMIOX	GCGTCAATCACATCAACCAAACTTT	GCTCATCTCCACCTTGTCCACTT
CsGalUR	GAGCAGCCTCTTGGAGAAGCAAT	ATCACGATGAGCATCAGAACACCAA
CsAO	CCAACACCACTCAAGCACTAACAATAC	GAGGATGATACGGCGGTGATGG
CsDHAR1	ATGATGGAACCGAGCAAGCATTACT	GACAAGTCCGCAGCAGATACTCTT
CsDHAR2	ACCCTCCTCTCTGCCATTCTCC	TTCATCCAGTGCCTTCAACTCATCAA
CsGR	ACCCTGATGGCTAATAAGAATGCTGAA	TAGTATGTGCCTTGCCGAGTAGAGT
CsMDAR	AGACTCTCGTTAGTGCTGCTGGA	TCTTCGCCTGAATTGCTTCTACAAGT
Csactin	GATTCCGTTGCCCTGAAGTCCT	CCTTGCTCATACGGTCTGCGATA

Statistical analysis

The mean value of three technical replicates was calculated. Differences in expression levels were determined using the Duncan multiple-range test at a 0.05 significance level in SPSS17.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Expression of AsA biosynthesis-related genes under high temperature stress in tea plants

The expression profiles of AsA biosynthesis-related genes under high temperature treatment had different patterns in the two tea plant varieties (Figure 2). In 'Yingshuang', the genes quickly responded to heat stress, and their expression levels increased substantially at 1 h. The expression levels of *CsPMM*, *CsGGP*, and *CsGalDH* were initially upregulated, and were then similar to those of the untreated plants under heat treatment in 'Yingshuang'. The expression levels of *CsGGP*, *CsGPP*, *CsGMP*, and *CsGME* changed little until 4 h in 'Huangjinya'. In contrast, the expression level of *CsPGI1* was downregulated during heat treatment until 12 h in 'Huangjinya'.

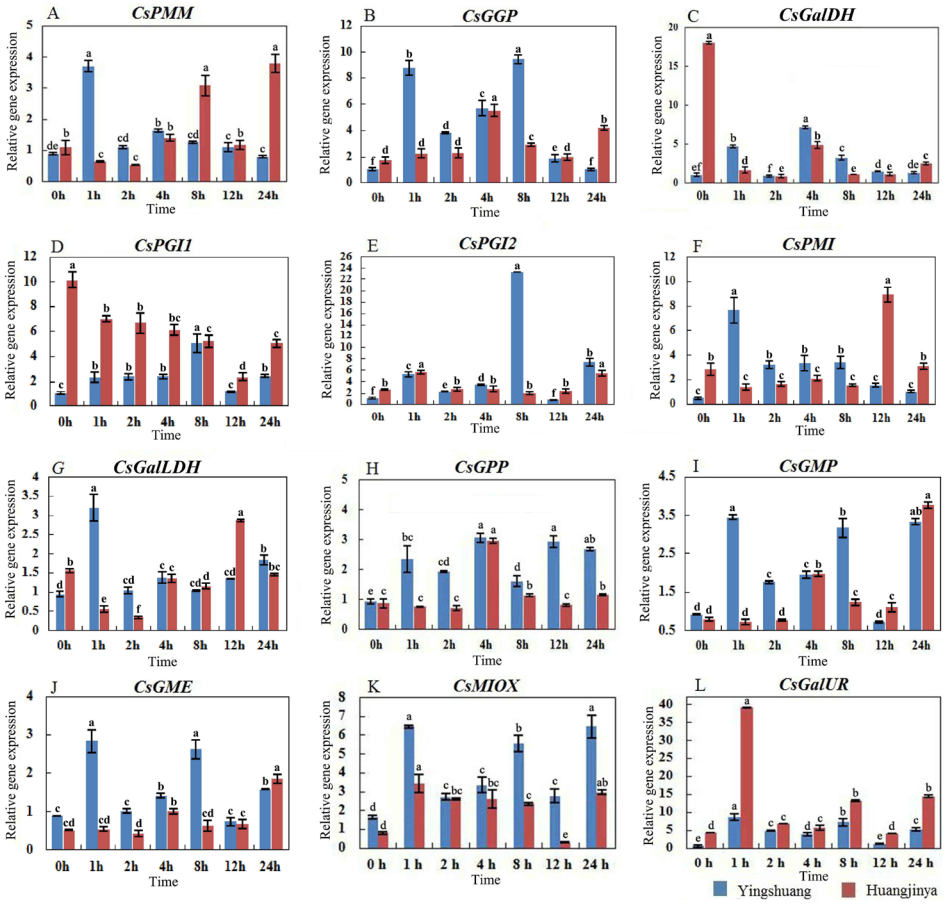

Figure 2. Expression analysis of genes involved in the ascorbic acid biosynthesis pathway in tea plants under high temperature. **A.** *CsPMM*, **B.** *CsGGP*, **C.** *CsGalDH*, **D.** *CsPGI1*, **E.** *CsPGI2*, **F.** *CsPMI*, **G.** *CsGalLDH*, **H.** *CsGPP*, **I.** *CsGMP*, **J.** *CsGME*, **K.** *CsMIOX*, **L.** *CsGalUR*. Error bars represent standard deviations of the means of three technical replicates.

Expression of AsA biosynthesis-related genes under low temperature stress in tea plants

In response to cold stress, the expression levels of *CsPGI1* in the two tea plant varieties were upregulated, peaked at 4 h, and then decreased (Figure 3). Although the expression trends of some genes related to AsA biosynthesis were similar, the expression levels of other genes in the two tea plant varieties under cold treatment significantly differed. The expression levels of *CsPMM* were similar in the two varieties at low temperature before 8 h, and had maximum and minimum values at 12 h in 'Huangjinya' and 'Yingshuang', respectively.

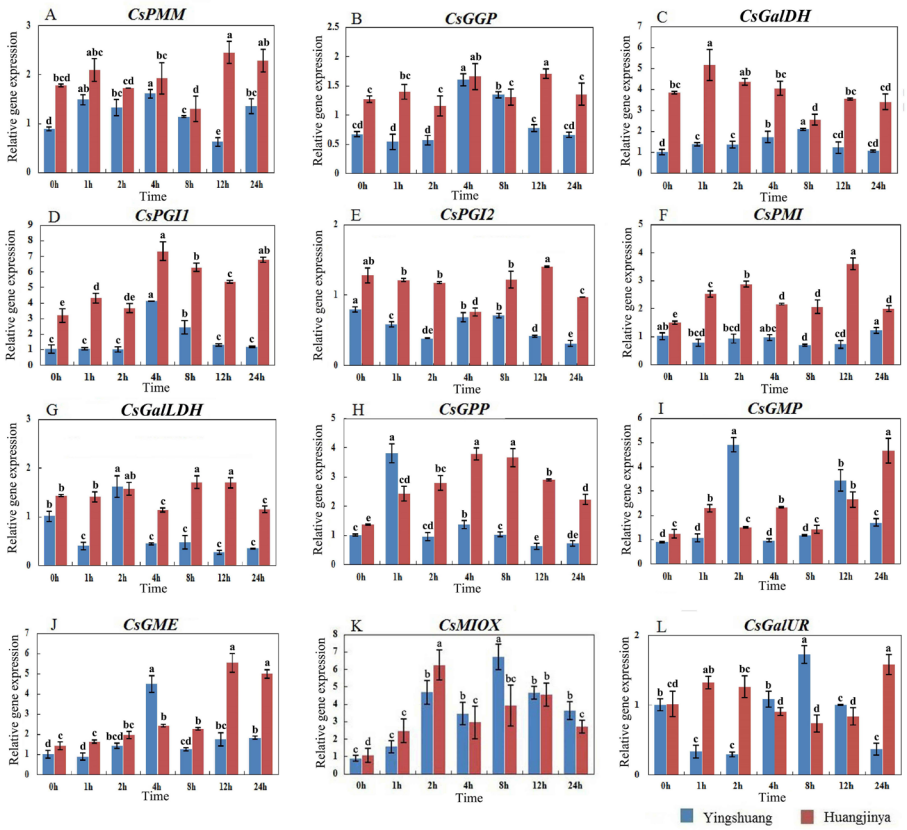

Figure 3. Expression analysis of genes involved in the ascorbic acid biosynthesis pathway in tea plants under low temperature. **A.** *CsPMM*, **B.** *CsGGP*, **C.** *CsGalDH*, **D.** *CsPGI1*, **E.** *CsPGI2*, **F.** *CsPMI*, **G.** *CsGalLDH*, **H.** *CsGPP*, **I.** *CsGMP*, **J.** *CsGME*, **K.** *CsMIOX*, **L.** *CsGalUR*. Error bars represent standard deviations of the means of three technical replicates.

Expression of genes involved in AsA recycling under high temperature stress in tea plants

In response to high temperature, the expression levels of *CsMDHAR*, *CsDHAR1*, and *CsDHAR2*, which are involved in AsA recycling in 'Yingshuang', increased at 1 h. In contrast,

the expression levels of these genes in 'Huangjinya' gradually increased during heat treatment (Figure 4). In 'Huangjinya', the expression levels of *CsAO* and *CsGR* were upregulated, and peaked at 12 and 8 h, respectively.

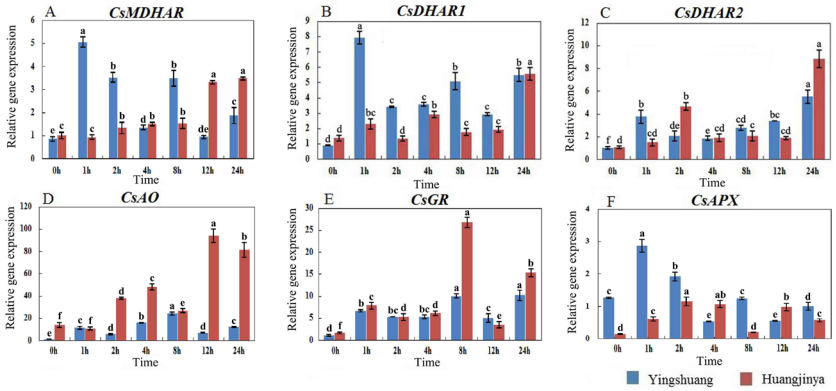

Figure 4. Expression analysis of genes involved in the ascorbic acid recycling pathway in tea plants under high temperature. **A.** *CsMDAR*, **B.** *CsDHAR1*, **C.** *CsDHAR2*, **D.** *CsAO*, **E.** *CsGR*), **F.** *CsAPX*. Error bars represent standard deviations of the means of three technical replicates.

Expression of genes involved in AsA recycling under low temperature stress in tea plants

The expression levels of *CsDHAR1* and *CsDHAR2* were similar during the low temperature treatment in 'Yingshuang'. The expression level of *CsDHAR1* initially increased and peaked at 4 h, before being downregulated. However, the expression patterns of *CsDHAR1* and *CsDHAR2* differed from 4 to 24 h in 'Huangjinya' under cold treatment (Figure 5). The expression levels of *CsAO* and *CsGR* in 'Yingshuang' were the same: they initially decreased and then increased.

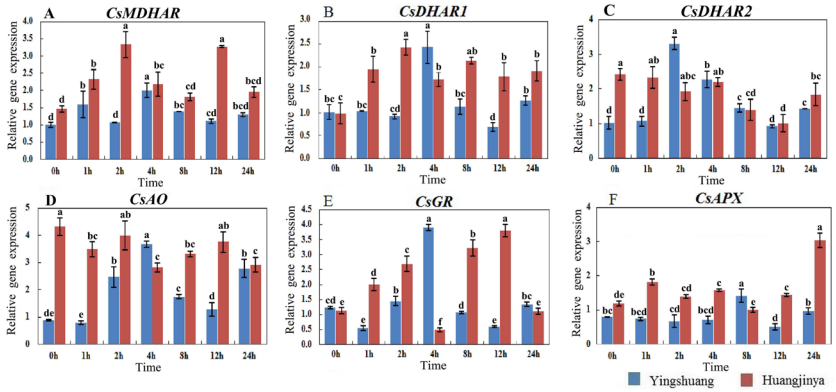

Figure 5. Expression analysis of genes involved in the ascorbic acid recycling pathway in tea plants under low temperature. **A.** *CsMDAR*, **B.** *CsDHAR1*, **C.** *CsDHAR2*, **D.** *CsAO*, **E.** *CsGR*, **F.** *CsAPX*. Error bars represent standard deviations of the means of three technical replicates.

DISCUSSION

Oxidative stress caused by the accumulation of ROS is an important cause of temperature stress-related damage in plants (Ali et al., 2005). As efficient ROS scavengers, several AsA metabolism-related enzymes, such as APX and MDHAR, play important roles in plant defense against extreme temperature stress (Almeselmani et al., 2006; Li et al., 2010; Massot et al., 2013). Previous studies have demonstrated that enzymes involved in AsA biosynthetic pathways, such as GME (Gilbert et al., 2009), GGP (Bulley et al., 2009), and MIXO (Lorence et al., 2004), may influence AsA levels.

Under heat stress, the expression level of *CsGGP* gradually increased, peaked at 4 h, and then decreased in 'Huangjinya'. A previous study reported a correlation between the expression levels of *VTC*, which encodes GGP, and the response to artificial inoculation in the grapevine (Hou et al., 2013). In the present study, the expression levels of *CsGMP* and *CsGME* were similar. This indicates that the transcriptional regulation of *CsGMP* and *CsGME* is similar; these genes are located upstream in the AsA biosynthesis pathway, and they may be the limiting factors of genotype differences under heat stress. The expression level of *CsGalLDH* in 'Yingshuang' peaked at 1 h and then decreased under high temperature stress. *GalLDH* is located on the mitochondrial inner membrane (Siendones et al., 1999). *CmGalLDH* expression in the melon (*Cucumis melo* L.) is controlled by light, and is higher in light-grown seedlings than in those grown in the dark (Pateraki and Kanellis, 2004). This suggests that the *CsGalLDH* expression pattern observed may have been in response to mitochondrial damage caused by high temperature stress.

The overexpression of *GME*s improves the tolerance of transgenic plants to cold stress in tomato (Zhang et al., 2011). The expression peaks of *CsGME* in 'Yingshuang' and 'Huangjinya' appeared at 4 and 12 h under low temperature, respectively. This finding indicates that *CsGME* may play a role in responding to environmental stress after a variable period. In higher plants, AsA degradation and recycling mechanisms are important for AsA accumulation (Gallie, 2013); DHAR and MDHAR are involved in the recycling process. APX, GR, DHAR, and MDHAR are important enzymes in the ascorbate-glutathione cycle, and regulate ROS levels under environmental stress (Lorence et al., 2004; Ma et al., 2008). AO mainly functions by adjusting the redox state of AsA outside the protoplasm of plants, and may respond to various environmental stressors (Pignocchi et al., 2003). Our data show that genes involved in AsA recycling were induced by heat and cold stress.

The overexpression of *APX* enhances the tolerance to chilling stress in transgenic tobacco plants (Yabuta et al., 2002). Two types of polymorphism have been found in the ascorbate peroxidase isozyme profile, with and without a cytosolic isoform (APX1). Cultivars that lack APX1 are more tolerant to chilling temperatures than those that have APX1 in soybean (Funatsuki et al., 2003). The transgenic *Arabidopsis* plants hosting *HvAPX1* gene exhibit a higher tolerance to heat stress than do wild-type plants (Shi et al., 2001). The expression levels of *CsAPX* were upregulated under cold and heat stress in 'Huangjinya', and they increased remarkably in response to high temperature in 'Yingshuang'. These results indicate that APX may play a major role in inducing resistance to temperature stress.

The expression levels of *MDHAR* and *DHAR* in acerola (*Malpighia glabra*) are regulated under abiotic stress (Urano et al., 2000; Eltelib et al., 2011). The mRNA abundances of *DHAR*, *APX*, and *GR* increase in wheat after acclimation to low temperature (Baek and Skinner, 2003), and a similar trend was reported in apple leaves in response to

high temperature (Ma et al., 2008). In 'Yingshuang', remarkable increases in the expression levels of *CsMDHAR*, *CsDHAR1*, and *CsGR* were observed at 1 h under heat treatment, which reached their maximum expression levels at 4 h under cold treatment. This indicates that the genes involved in the AsA recycling pathway also elicit responses to temperature stress, because their expression patterns differed under high and low temperature stress. *CsGalLDH* and *CsAPX* may play similar roles in responding to temperature stress.

The expression patterns of the AsA-related genes depended upon the cultivar used and the temperature treatment. Overall, our findings provide new insights into the molecular breeding and stress tolerance of the tea plant, and could be used to increase the AsA content and quality of tea plant leaves. In addition, transcriptomics, proteomics, metabolomics, functional genomics, and the full-genome sequencing of the tea plant will further improve our understanding of the processes and mechanisms involved in AsA biosynthesis and recycling in this species.

Conflicts of interest

The authors declare no conflict of interest.

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