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ORIGINAL ARTICLE

Molecular cloning and characterization of a γ-glutamylcysteine synthetase gene from *Chorispora bungeana*

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Abstract *Chorispora bungeana* Fisch. and C.A. Mey (*C. bungeana*) is a rare alpine subnival plant species that is highly tolerant of freezing temperatures. Glutathione (GSH) is a major player in various metabolic processes involved in plant growth and development and stress responses. A recent study has found that the inhibition of GSH synthesis decreases the chilling tolerance of *C. bungeana* callus (J. Wu et al.: Cryobiology 57:9–17, 2008). We have isolated and characterized a full-length cDNA encoding γ-glutamylcysteine synthetase (CbγECS), the key enzyme of GSH synthesis, from the leaves of *C. bungeana*, with the aim of furthering our understanding of the role of GSH at the molecular level. CbγECS was found to encode a 524-amino acid protein with moderate to high nucleotide sequence similar to previously reported plant γ-ECS genes. CbγECS transcripts were detected in the calluses, roots and leaves of *C. bungeana*, but there was no tissue-specific expression. The transcripts of CbγECS accumulated both rapidly and at high levels when *C. bungeana* was treated with chilling (4°C), freezing (−4°C), several other environmental stresses (such as heat, salinity, osmotic and heavy metals), abscisic acid and hydrogen peroxide. These results suggest that CbγECS may participate in the cellular responses against multiple environmental stimuli in *C. bungeana*.

Keywords Abscisic acid · Cold treatment · *Chorispora bungeana* · γ-Glutamylcysteine synthetase · Stress response

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Abbreviations

ABA	Abscisic acid
GSH	Glutathione
γ-ECS	γ-Glutamylcysteine synthetase
RACE	Rapid amplification of cDNA ends
RT-PCR	Reverse transcription–PCR

Introduction

Glutathione (GSH; γ-glutamylcysteinyl glycine) is an abundant and ubiquitous thiol with proposed roles in the storage and transport of reduced sulphur, the synthesis of proteins and nucleic acids and modulation of enzyme activity (May et al. 1998a, b; Noctor et al. 1998). In plants, GSH has been found to have multiple functions during plant growth and development, such as the regulation of cell division, root hair growth, tracheary element differentiation, flowering, anthocyanin accumulation and the regulation of sugar metabolism (Sanchez-Fernandez et al. 1997; Vernoux et al. 2000; Henmi et al. 2001; Ogawa et al. 2001; Xiang et al. 2001; Ito et al. 2003; Ogawa et al. 2004). It also plays important roles in the adaptation of plants to biotic and abiotic stresses (May et al. 1998a, b), with salt stress, heavy metal exposure, pathogens and low temperature all leading to GSH accumulation (Kocsy et al. 1996, 1997, 2000; Ruiz and Blumwald 2002; Mittova et al. 2003).

Glutathione is synthesized from its constituent amino acids in an adenosine triphosphate (ATP)-dependent, two-step reaction catalyzed by the enzymes γ-glutamylcysteine synthetase (γ-ECS) and glutathione synthetase. Recent studies have shown that in the Brassicaceae plant family γ-ECS is localized exclusively in the plastids, whereas glutathione synthetase is found both in plastids and in the

cytosol (Noctor et al. 2002; Wachter et al. 2005). The biosynthesis of GSH has been extensively studied in transformed plants, with the results revealing that GSH synthesis is controlled by the level of γ-ECS activity, the availability of the substrate and the feedback inhibition of γ-ECS by GSH (Noctor et al. 1998). Bioinformatics analysis of the γ-ECS genes from multiple species suggests that these sequences can be grouped into three families: sequences from the γ-proteobacteria; sequences from non-plant eukaryotes; sequences from plants and α-proteobacteria. Sequence comparisons within each family show some degree of similarity, but pairwise comparisons between groups have revealed the absence of statistically significant relationships (Copley and Dhillon 2002). For example, *Arabidopsis thaliana* γ-ECS (AtγECS) shares less than 15% amino acid sequence identity with the members of other families (May and Leaver 1994). The differences among γ-ECS sequences also reflect the functional properties of each family.

Chorispora bungeana Fisch. and C.A. Mey (*C. bungeana*) is a representative alpine subnival plant which can survive chilling, even freezing, temperatures and frequent temperature fluctuations. This plant is a perennial herb belonging to Brassicaceae. It grows in the freeze–thaw tundra at the edges of glaciers, a habitat receptive to very few other flowering plants. Its main distribution is within an ice-free cirque (with a 3800–3900 m a.s.l.) beside Glacier No. 1 in the source area of the Urumqi River in Tianshan Mountains, Xinjiang province, China. The temperature conditions consist of chilling-temperatures weather during the day and freezing-temperatures during the night, with the occasional snow or hail storm during the growth period in the summer. Over the years, much effort has been expended in attempts to determine the chilling- and freezing-tolerance mechanisms of this plant. It does not appear to possess special morphological characteristics for an adaption to a cold environment (Ayitu et al. 1998; An et al. 2000), suggesting that certain physiological and molecular mechanisms must be contributing to its successful adaptation to environmental stress. For example, in *C. bungeana*, the distribution and accumulation of Ca^{2+} in response to cold may play an important role in its active cold-tolerance at 0°C. Also, during cold treatment (including 4, 0 and −4°C), the redox transition of ubiquinone can ensure the fluency of electron transfer in mitochondria and facilitate the regulation of the whole-cell redox states, suggesting that cold-tolerance in this plant is tightly linked with the redox balance of its cellular redox molecule (Chang et al. 2006; Fu et al. 2006).

We have recently found that the inhibition of GSH synthesis decreases the chilling tolerance in *C. bungeana* callus by affecting the unsaturated fatty acid compositions and fluidity and enzymatic activity of the plasma membrane (PM) (Wu et al. 2008). These results suggest that GSH plays a pivotal role in the low-temperature tolerance of *C. bungeana*. In order to further our understanding of the role of GSH at the molecular level, we cloned and characterized the γ-glutamylcysteine synthetase gene from *C. bungeana*, CbγECS. In a second step aimed at obtaining some insight into its behavior and regulation by environmental stimuli, we characterized the mRNA expression profile of CbγECS against several environmental stresses (such as heat, salinity, osmotic and heavy metals), abscisic acid (ABA) and hydrogen peroxide (H_2O_2). The results obtained in our study demonstrate that the transcript accumulation of CbγECS is regulated by multiple environmental stresses.

Materials and methods

Plant materials and growth conditions

Seeds and leaves of *C. bungeana* were collected from an ice-free cirque (43°05′N, 86°49′E, altitude 3800–3900 m a.s.l.) near the No.1 glacier in the source area of Urumqi River in Tianshan Mountains, Xinjiang, China. Embryogenic callus derived from mature seeds of *C. bungeana* and regenerated plants were obtained as described by Fu et al. (2006) with some modifications. Briefly, the seeds were rid of their hard coats with sand paper, rinsed in 70% ethanol for 30 s, surface sterilized in 1% hypochlorite solution plus 0.1% Tween-20 for 20 min and washed three times with sterile water. The cotyledons were cut off and placed on MS (Murashige and Skoog 1962) medium supplemented with 1 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.2 mg/L 6-benzyladenine (6-BA), then cultured at 25°C under a 16/8-h (ligh/dark) photoperiod with light supplied at an irradiance of 80 μmol m^{-2} s^{-1}. After 20 days, the embryogenic calluses were picked out for sub-culturing on MS medium supplemented with 0.5 mg/L 2, 4-D, 0.1 mg/L 6-BA and 0.5 mg/L α-naphthalene acetic acid (NAA). Following approximately six sub-cultures in the same medium, the calluses were used for experiments. Regenerated plants were induced by transferring the calluses to MS medium containing 0.4 mg/L gibberellin (GA_3), 0.6 mg/L kinetin (KT) and 3% glucose instead of 3% sucrose. The seedlings were sub-cultured once a month. When the regenerated plants from the second subculture were approximately 4 cm tall, they were used for experiments.

Stress treatments

Calluses were used as experimental material in experiments involving different stress conditions. Cold treatments consisted of transferring calluses to a growth chamber set at 4°C or −4°C for a maximum of 9 days. The calluses were collected at various time points during the cold treatment.

For the ABA treatment, a final concentration of 100 μ*M* filter-sterilized ABA was added to the solid medium for 48 h. For the H_2O_2 treatment, 2 mL filter-sterilized 5 m*M* H_2O_2 was added once every 24 h onto the surface of solid medium and the calluses exposed to the H_2O_2 for 48 h. In the heat treatment, calluses were placed in growth chamber maintained at 40°C for 48 h. For the osmotic treatment, 2 mL filter-sterilized 20% PEG6000 (polyethylene glycol) was added onto the surface of solid medium. Other stress treatments included adding 200 m*M* NaCl, 100 μ*M* $CdCl_2$ or 100 μ*M* $CuCl_2$ directly to the solid medium before autoclaving. After all treatments, the calluses were collected and washed three times in distilled water, and the excess water was blotted with filter paper. The samples were analyzed immediately or immersed rapidly in liquid N_2 for subsequent RNA isolation.

Cloning of the middle region of the CbγECS

Total RNA was isolated directly from 0.1 g *C. bungeana* leaves using Trizol reagent (Invitrogen, Carlsbad, CA). Using the structure of published genes as a basis, we designed degenerated primers to conserved γ-ECS and *Arabidopsis thaliana*, *Brassica juncea*, *Lycopersicon esculentum*, *Medicago truncatula*, *Phaseolus vulgaris*, *Pisum sativum* and *Zea mays*. Total RNA was used to synthesize the first strand of cDNA with Superscript II RT. The cDNA was used as a template and the SenseP and AntiP as primers (reference to Table 1) in a 35-cycle PCR (annealing temperature 52°C) to amplify partial γ-ECS cDNA. The PCR product was cloned into pGEM-T vector (Promega, Madison, WI) and sequenced.

5′-Rapid amplification of cDNA ends of CbγECS

The first strand cDNA was synthesized according to the manufacturer's guidelines of the SMART rapid amplification of cDNA ends (RACE) cDNA amplification kit (Clontech, Palo Alto, CA) using the PowerScript reverse transcriptase, SMART II An oligonucleotide and 5′-RACE CDS primer provided with the kit. The first PCR was performed with the UPM provided with the kit as the forward primer and the gene-specific primer 5′GSP as the reverse primer. The PCR was carried out by first denaturing the cDNA at 94°C for 3 min followed by 35 cycles of amplification (94°C for 30 s, 64°C for 30 s, 72°C for 60 s) and a final extension at 72°C for 5 min. The nested PCR was then performed with the internal primer of the UP primer (NUP) provided with the kit as the forward primer and the nested gene-specific primer 5′NGSP as the reverse primer with annealing temperature of 55°C. The PCR product was purified, cloned into pGEM-T vector and sequenced.

Table 1 All primers used in amplification of *Chorispora bungeana* γ-glutamylcysteine synthetase (CbγECS)

Primers	Sequence
SenseP	5′-ATTGT(A/T)GC(G/T)GC(A/G/T)AG(C/T)CCTC C(A/C)AC-3′
AntiP	5′-CTC(A/C)GC(A/C/T)GG(A/C/T)GT(A/C/G/T)AC (A/T)CCTGTT-3′
UMP	long, 5′-CTAATACGACTCACTATAGGGCAAGCA GTGGTATCAACGCAGAGT-3′ and short, 5′-CTAATACGACTCACTATAGGGC-3′
NUP	5′-AAGCAGTGGTATCAACGCAGAGT-3′
5′GSP	5′-GGGCGTAAAGTATTGACGTCGAAACC-3′
5′NGSP	5′-TACATCCAGAGGCAAGATAGGCA-3′
3′GSP	5′-GTCGCTGAAGATGTCCTGAAACT-3′
Act-FP	5′-GCTCCGTGTTGCCCCTGAAGA-3′
Act-RP	5′-CTCGGCGGTGGTGGTGAACA-3′
RT-FP	5′-TGACTATGCACTTGATGTCCCTAT-3′
RT-RP	5′-AAACCAGCTTCTTTGTAGCCTCT-3′

RT, Reverse transcription; FP, forward primer; RP, reverse primer; Act, Actin; NGSP, nested gene-specific primer; NUP, internal primer of UP primer

3′-RACE and full-length cDNA cloning of CbγECS

The first strand cDNA was synthesized according to the manufacturer's guidelines of the SMART RACE cDNA Amplification kit (Clontech) using the PowerScript reverse transcriptase and 3′-RACE CDS primer A provided with the kit. The 3′-RACE–PCR was performed with the gene-specific primer 3′GSP as the forward primer and the NUP provided with the kit as the reverse primer. The PCR was carried out by first denaturing the cDNA at 94°C for 3 min followed by 35 cycles of amplification (94°C for 30 s, 56°C for 30 s, 72°C for 60 s) and a final extension at 72°C for 5 min. The PCR product was then purified and cloned into pGEM-T vector and sequenced. By comparing and aligning the sequences of 3′RACE, 5′-RACE and the middle region products, we were able to obtain the full-length cDNA sequence of CbγECS. The full-length CbγECS was subsequently characterized at the molecular level, such as the putative protein sequence and sequence homology.

Semi-quantitative RT-PCR analysis

To investigate the tissue-specific expression pattern of CbγECS and the expression patterns of CbγECS under different stresses, we extracted total RNA from 0.1 g *C. bungeana* calluses, regenerated plant leaves or roots using the Trizol reagent and subjected the total RNA samples to RT–PCR analysis. Aliquots of 1 μg total RNA of each treatment were used as the template in RT-PCR reactions with the RT-FP (forward primer) and RT-RP (reverse primer) as primers in a one-step RT-PCR kit (Takara,

Japan). The numbers of cycles in the PCR were varied in order to establish a point at which no band saturation occurred. Amplifications were performed at 94°C for 3 min followed by 27 cycles of amplification (94°C for 40 s, 52°C for 40 s, 72°C for 40 s). PCR products (5 μL) were separated on 1% agarose gels and stained with ethidium bromide. The bands of the PCR products were analyzed by Gene Tools software from the Gene Company (Hong Kong). The RT-PCR for *actin* (GenBank Accession No. AY825362), using specific primers Act-FP and Act-RP (Zhang et al. 2006), was performed under the same conditions as described above to estimate if equal amounts of RNA among samples were being used in the RT-PCR reaction. The RT-PCR reactions were repeated at least three times, and representative results are shown in the figures shown in the Results

Results

Cloning and sequence analysis of the full-length cDNA of CbγECS

Using known cDNA sequences of the conserved regions of plant γECS genes, we designed and synthesized primers SP and AntiP for the amplification of the middle region of γ-ECS-like cDNA from *C. bungeana*. A single fragment of about 1300 bp was obtained. Based on the middle region sequence, we designed two reversed gene-specific primers, 5′GSP and 5′NGSP, for the 5′-RACE. After two PCR amplifications, we obtained a single and specific fragment of about 350 bp in which a 15-bp 5′ untranslated region (UTR) was found upstream of the first ATG codon. A gene-specific primer, 3′GSP, was designed and used in the 3′-RACE, resulting in a single and specific fragment of about 400 bp; a 185-bp 3′UTR was found downstream from the stop codon. Based on the sequences of the middle region sequence and the 5′- and 3′-RACE products, we were able to deduce the full-length cDNA. The full-length cDNA sequence and deduced amino acid sequence of CbγECS are presented in Fig. 1. The 1575-bp open reading frame (ORF) encodes a predicted protein of 524 amino acids with a calculated molecular weight of about 58.8 kDa and a pI of 6.5. The predicted protein contains a putative plastidic transit peptide of 75 amino acids. (GenBank accession No. EF137428).

The predicted amino acid sequences of CbγECS were aligned with the cloned γECS from various plants using DNAStar software (Madison, WI). A phylogenetic tree, based on the genetic distance of the protein sequences as determined by the Clustal method using DNAStar software, is shown in Fig. 2a and b. A comparison of the predicted protein sequences of the CbγECS with γ-ECS of other plants shows that CbγECS is highly identical to γ-ECS from *A. thaliana* (94%), *B. juncea* (93%), *L. esculentum* (78%), *M. truncatula* (78%), *O. sativa* (74%), *P. vulgaris* (79%), *P. sativum* (80%) and *Z. mays* (80%) (Fig. 2a and b).

Expression patterns of CbγECS in different tissues and under abiotic stresses

To investigate CbγECS patterns in various tissues of *C. bungeana*, we isolated total RNA from the calluses and the leaves and roots of regenerated plants and subjected it to semi-quantitative RT-PCR analysis. The results indicated that CbγECS is ubiquitously expressed in *C. bungeana*, with almost no tissue specificity even though the expression levels showed slight variations between tissue types (Fig. 3).

The accumulation pattern of the CbγECS mRNA in response to low temperature was studied by means of a semi-quantitative RT-PCR analysis of total RNA extracted at different times after the initiation of the cold treatment. Following exposure to 4°C, the transcript levels of CbγECS progressively increased, almost reaching a plateau after 5 days of treatment (Fig. 4a). After exposure to −4°C, the transcript level of CbγECS also increased rapidly, similar to that observed at 4°C, and the level of the CbγECS transcript remained at a high level even after 9 days of treatment (Fig. 4b).

The results of several previous studies indicate that low temperature significantly elevates the endogenous levels of ABA and that the increased levels of ABA may be involved in a number of stress-responsive gene expression and stress tolerance mechanisms (Xiong et al. 2002). Since the transcript levels of CbγECS increased in *C. bungeana* in response to low temperature, the effect of this phytohormone was also tested. We found that the transcript levels of CbγECS progressively increased from 6 to 48 h of treatment with 100 μ*M* ABA (Fig. 5a). The generation of H_2O_2 is induced in plants following exposure to a wide variety of abiotic and biotic stimuli (Neill et al. 2002a, b). Excess H_2O_2 may induce oxidative stress in plants, but it is also evident that H_2O_2 can function as a signaling molecule. To investigate whether CbγECS transcript levels respond to exogenous H_2O_2, we applied 5 m*M* H_2O_2 to *C. bungeana* medium. The transcript levels barely changed during the first 12 h of the treatment, but they increased markedly compared with control after 48 h of treatment (Fig. 5b).

Plant CbγECS have been shown to be regulated at the transcript level by several stimuli, suggesting some lack of specificity in the transcriptional response of CbγECS. The effects of several other environmental conditions were also examined. As shown in Fig. 6, the expression of CbγECS was also induced by exposure to several treatments in addition to low temperature (4 and −4°C). The heat (42°C), NaCl and PEG6000 treatments strongly induced the

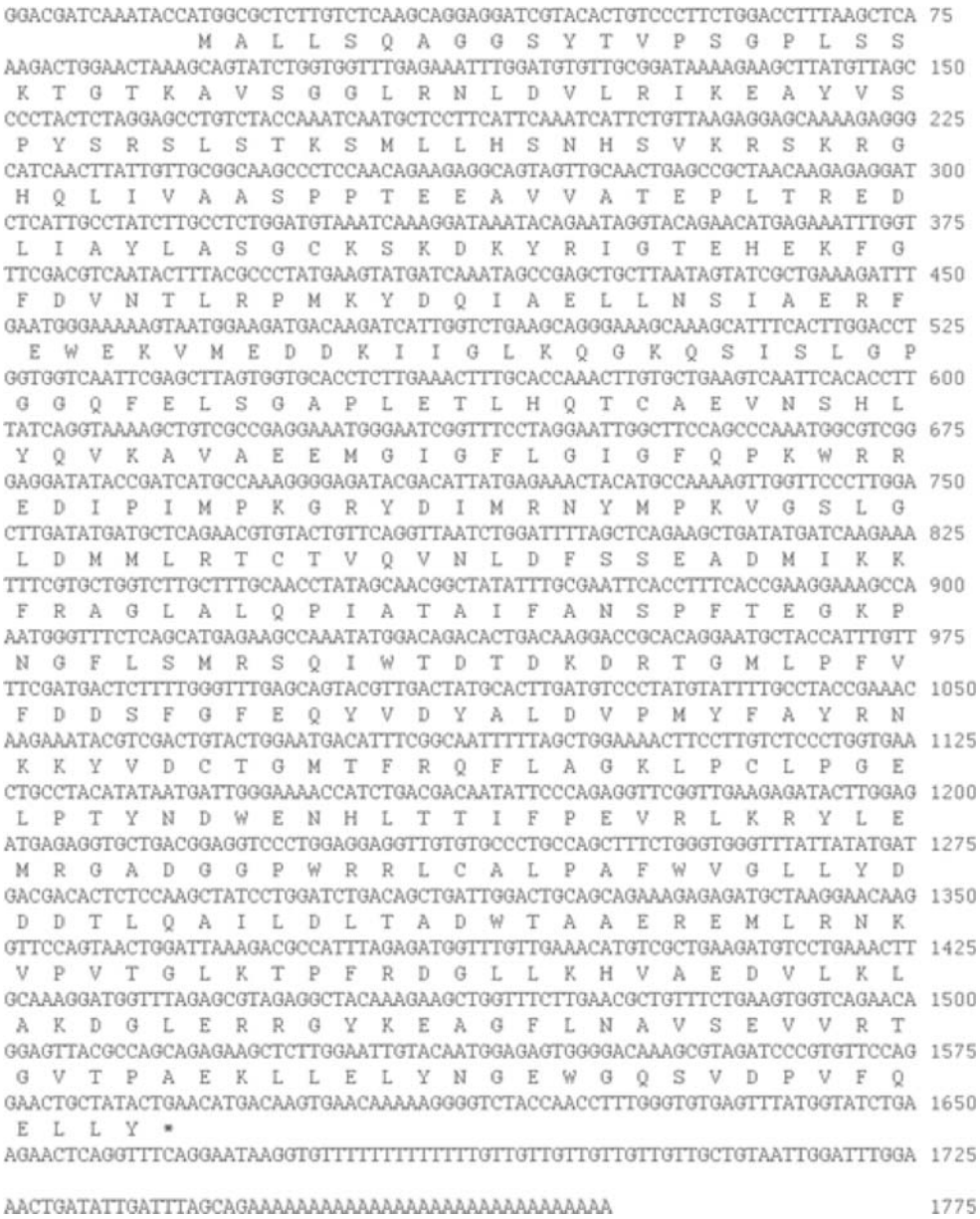

Fig. 1 The full-length cDNA sequence and deduced amino acid sequence of *Chorispora bungeana* γ-glutamylcysteine synthetase (*CbγECS*) cDNA (GenBank accession No. EF137428). The nucleotides are numbered on the right

expression of CbγECS. Exposure to heavy metals (Cu^{2+} and Cd^{2+}) also induced CbγECS expression. These results revealed that CbγECS may be regulated by multiple environmental stresses.

Discussion

In plants, GSH is an important molecular player involved in plant growth and development and stress responses (Ogawa 2005). GSH detoxifies reactive oxygen species via the ascorbic acid–GSH cycle (Foyer and Noctor 2005) and glutathione peroxidases. It is also involved in the detoxification of xenobiotics via glutathione S-transferases (Edwards et al. 2000; Dixon et al. 2002) and serves as a major defense component against a wide range of abiotic and biotic stress factors. Recent studies have shown that GSH can act as a signaling molecule to post-translationally modify proteins via glutathionylation (Dixon et al. 2005; Michelet et al. 2005). The catalytic properties of plant recombinant γ-ECS, the key enzyme of GSH biosynthesis, have been studied in *Arabidopsis* (Jez et al. 2004), and the regulation of this plant enzyme is well understood in *B. juncea* (Hothorn et al. 2006). However, the regulation of transcript levels of γ-ECS is still poorly understood, and there has been some debate over previous results. For example, it is known that γ-ECS mRNA levels in *Arabidopsis* leaves increase in response to heavy metals

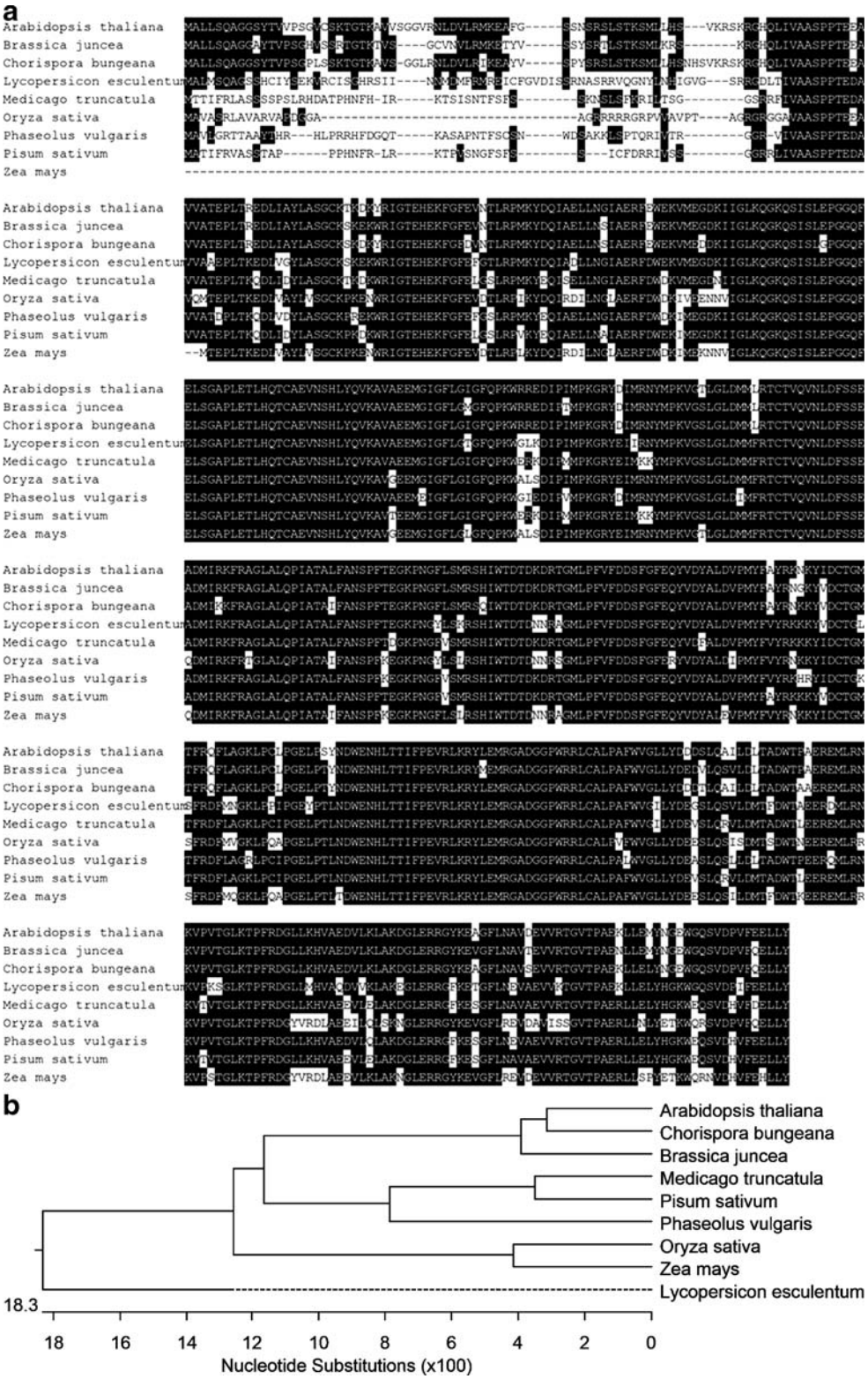

Fig. 2 **a** Multiple alignment of the *C. bungeana* γ-ECS (*CbγECS*) with γ-ECS from other plant species,**b** phylogenetic analysis of γ-ECS from various plants. The phylogenetic tree is based on the genetic distance of protein sequences and was constructed by the Clustal method using DNAstar software. The plant γ-ECS used for alignment and construction of the tree are: *Arabidopsis thaliana* γ-ECS (GenBank Accession No. Y09944) *Brassica juncea* γ-ECS (GenBank Accession No. Y10848), *Lycopersicon esculentum* γ-ECS (GenBank Accession No. AF017983), *Medicago truncatula* γ-ECS (GenBank Accession No. AF041340), *Oryza sativa* γ-ECS (GenBank Accession No. NM_001061076), *Phaseolus vulgaris* γ-ECS (GenBank Accession No. AF128454), *Pisum sativum* γ-ECS (GenBank Accession No. AF128455) and *Zea mays* γ-ECS (GenBank Accession No. AJ302783)

and jasmonic acid (Xiang and Oliver 1998), but that AtγECS levels in *Arabidopsis* suspension cells are not affected by exposure to heavy metals (May et al. 1998a, b).

We cloned the γ-ECS gene from *C. bungeana*, a representative alpine subnival plant, and denoted it as CbγECS. Identity analysis showed that CbγECS is identical to γ-ECS from other plants, especially that from *A. thaliana* (94%) and *B. juncea* (93%). (Fig. 2a). The conspicuous diversity of the amino acid sequences of γ-ECS was pointed out by Copley and Dhillon (2002), and a

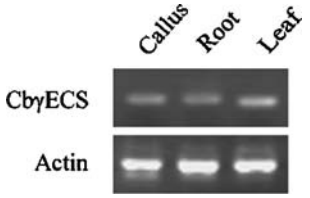

Fig. 3 Expression patterns of CbγECS in different tissues. Total RNA (1 μg per sample) was isolated from callus and the roots and leaves of regenerated plants and subjected to one-step reverse transcription (RT)-PCR amplification with 27 cycles (*upper panel*). The *actin* gene (GenBank Accession No. AY825362) was used as the control to show the normalization of the amount of templates in PCR reactions (*lower panel*)

molecular phylogenetic tree of the amino acid sequences of γ-ECS from prokaryotic and eukaryotic organisms has been constructed (Ashida et al. 2005). The results of these two studies indicate that α-proteobacterial γ-ECS (such as those from *Xanthomonas axonopoides* and *Clostridium crescentus*) are much closer to those from plants than expected, suggesting that plant γ-ECSs may have evolved from an α-proteobacterial gene. As shown in Fig. 2b, with the exception *L. esculentum*, the plant γ-ECS can be grouped

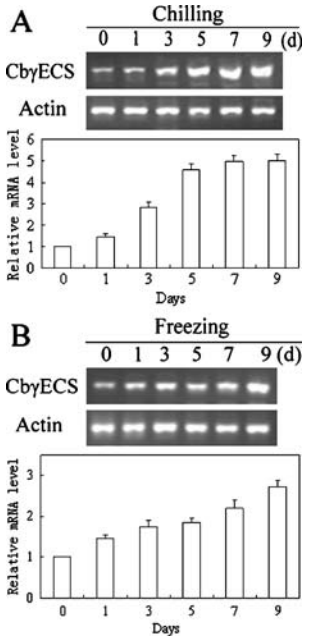

Fig. 4 Expression patterns and relative mRNA levels of CbγECS following exposure to chilling (4°C) (**a**) and freezing (−4°C) (**b**). **a** Calluses were treated at 4°C for 0, 1, 3, 5, 7 and 9 days. **b** Calluses were treated at −4°C for 0, 1, 3, 5, 7 and 9 days. For all of these treatments, total RNA (1 μg per sample) was isolated from calluses and subjected to one-step RT-PCR amplification with 27 cycles (*upper panel*). The *actin* gene was used as an internal control (*lower panel*). The mRNA levels were qualified with Imager 1D/2D software and normalized to the *actin* gene. Relative transcript levels were calculated with reference to the controls [taken as 1 (100%)]. The results are given as the mean ± standard error (SE) of three independent experiments

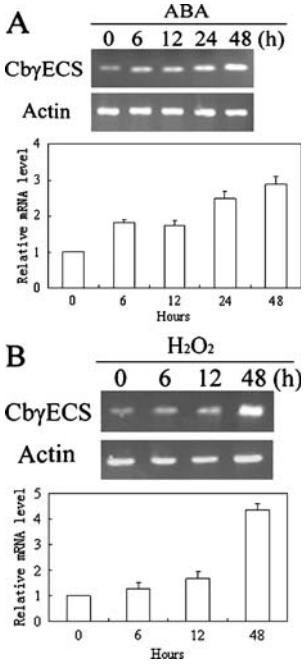

Fig. 5 Expression patterns and relative mRNA levels of CbγECS in response to abscisic acid (*ABA*) (**a**) and hydrogen peroxide (H_2O_2) (**b**). (**a**) For the ABA treatment, 100 μ*M* of filter-sterilized ABA was added onto the solid medium and the calluses picked out at 0, 6, 12, 24, 48 h. (**b**) For the H_2O_2 treatment, 5 m*M* filter-sterilized H_2O_2 was added once every 12 h onto the solid medium and the calluses picked out at 0, 6, 12, 48 h. For all of these treatments, total RNA (1 μg per sample) was isolated from the calluses and subjected to one-step RT-PCR amplification with 27 cycles (*upper panel*). The *actin* gene was used as an internal control (*lower panel*). The mRNA levels were qualified with Imager 1D/2D software and normalized to the *actin* gene. Relative transcript levels were calculated with reference to the controls [taken as 1 (100%)]. The results are means ± SE of three independent experiments

into two distinct groups, with the monocotyledonous plants constituting one group and the dicotyledonous plants constituting another.

Spatial expression analysis showed that CbγECS is expressed in different tissues of *C. bungeana* including calluses, roots and leaves (Fig. 3). A basal level of CbγECS transcripts is expressed, as GSH is required as a basal element in plant growth and development and serves as a major defense component against a wide range of abiotic and biotic stress factor.

Chorispora bungeana is a rare and typical alpine subnival plant growing near the No. 1 glacier, located at the origin of Urumqi River in the Tianshan Mountains of China. The annual average temperature is lower than 5°C during the day and −4°C at night. The temperature also fluctuates widely—from nearly 4 to −10°C—during the favorable growth season from June to September, and the plant can survive even in embedded in snow. The plant therefore demonstrates an extraordinary resistance to freezing temperatures. Previous research showed that lines

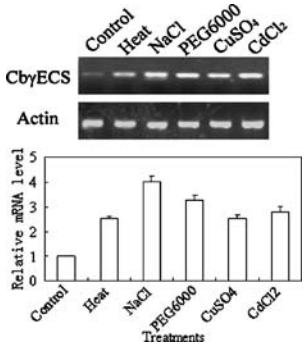

Fig. 6 Expression patterns and relative mRNA levels of CbγECS under various environmental stresses. Calluses were grown for 48 h on medium to which 200 m*M* NaCl, 20% PEG6000, 100 μ*M* $CdCl_2$ or 100 μ*M* $CuCl_2$ had been added or they were subjected to heat (40° C) for 48 h. For all of these treatments, total RNA (1 μg per sample) was isolated from calluses and subjected to one-step RT-PCR amplification with 27 cycles (*upper panel*). The *actin* gene was used as an internal control (*lower panel*). The mRNA levels were qualified with Imager 1D/2D software and normalized to the *actin* gene. Relative transcript levels were calculated with reference to the controls [taken as 1 (100%)]. Results are the means ± SE of three independent experiments

of calluses and suspension-cultured cells from *C. bungeana* can inherit the cold tolerant properties of this plant (Guo et al. 2006). Moreover, calluses grow uniformly and show a direct and rapid response to various experimental conditions, including those used in the study reported here. We therefore conducted our experiments using calluses to elucidate the function of CbγECS during the different stress treatments.

Wu et al. (2008) found that an inhibition of GSH synthesis decreased the chilling tolerance in *C. bungeana* callus by affecting the unsaturated fatty acid composition and fluidity and enzymatic activity of the PM. These results suggest that GSH plays a pivotal role in the low-temperature tolerance of *C. bungeana*. In order to further our understanding of the role of GSH at the molecular level, we investigated the expression pattern of CbγECS at different temperatures. After exposure to 4°C, transcript levels of CbγECS progressively increased, almost plateauing after 5 days of treatment (Fig. 4a). Gomez et al. (2004) also found that short-term chilling increased γ-ECS transcription in leaves of maize, but total γ-ECS protein expression and enzyme activities in the leaves were not increased. Maize, a C_4 species of subtropical origin, is cold sensitive and shows little or no cold acclimation traits. However, *C. bungeana* is a natural cold-resistant species (Chang et al. 2006; Fu et al. 2006; Guo et al. 2006). Our studies also revealed that cold was capable of increasing the enzyme activities of γ-ECS in *C. bungeana* (data not shown), suggesting that a different regulatory mechanism of γ-ECS may exist in *C. bungeana*. When *C. bungeana* was exposed to −4°C, the transcript levels of CbγECS increased rapidly, in a manner similar to that observed at 4°C, and they remained high even after 9 days of treatment (Fig. 4b). The expression patterns of CbγECS at different cold temperatures indicated that the induction of CbγECS by cold occurred by means of a time-dependent pathway. To our knowledge, this is the first description of a plant γ-ECS gene whose expression is inducible at 4°C and −4°C.

Most plant growth regulators are involved in plant stress tolerance, including cold stress. For example, salicylic acid may improve chilling tolerance in maize (Janda et al. 1999) as well as freezing tolerance (Mora-Herrera et al. 2005). Another plant growth regulator, ABA, also plays a crucial role in plant responses to chilling stress and cold acclimation. Cellular ABA accumulates in response to cold stress and increases in concentration can lead to a number of physiological adaptations, including stomata closure and growth inhibition, as well as up-regulation of cold-related genes. The GSH molecule plays important roles in the adaptation of plants to biotic and abiotic stresses, so GSH biosynthesis and metabolism may be regulated by plant growth regulators. Jasmonic acid treatment has been found to increase the expression of γ-ECS in *Arabidopsis* and the capacity for GSH synthesis but not to alter the GSH content in the unstressed plant (Xiang and Oliver 1998). Exogenous ABA significantly enhances the activity and expression levels of glutathione reductase (GR) in plants (Kaminaka et al. 1998; Contour-Ansel et al. 2006). In maize, chemically manipulated levels of GSH content and chilling tolerance were not correlated with changes in ABA levels (Kocsy et al. 2000), indicating that the protective effect of GSH during chilling stress is not dependent on ABA. However, these same results also suggest that ABA may act as an upstream molecular signal in the regulation of the gene for GSH biosynthesis and metabolism or the GSH pool. To date, however, it is unclear whether ABA can affect γ-ECS expression. In our study, exogenous ABA treatment increased the expression of CbγECS progressively from 6 to 48 h in *C. bungeana* (Fig. 5a), indicating that ABA is able to affect GSH biosynthesis and suggesting that GSH biosynthesis may occur through an ABA-dependent pathway under stress conditions. Whether ABA is involved in the observed increases of GSH induced by low temperature in *C. bungeana* requires further research.

The levels of H_2O_2 frequently increase in plants as a result of exposure to biotic and abiotic stresses, revealing the central role of this compound in the induction of defense genes (Vandenabeele et al. 2003; Mora-Herrera et al. 2005). H_2O_2 is considered to act as a signaling molecule because it is relatively stable and diffusible (Neill et al. 2002a, b). The effects of exogenous H_2O_2 application on plant stress tolerance are consistent with a signaling role. Gechev et al. (2002) reported that H_2O_2 treatments resulted in the experimental plant material showing a greater tolerance to

oxidative stress than untreated controls by inducing a set of antioxidant enzymes. In our study, H_2O_2 treatment did not affect the expression of CbγECS during a relative short time (12 h), which is consistent with a previous report in *Arabidopsis* (Xiang and Oliver 1998). However, after long time treatment (as much as 48 h), the transcript levels of CbγECS were obviously induced (Fig. 5b). These results may be due the fact that the short-time treatment only induced post-transcriptional regulation (May et al. 1998a, b), while the continuous long-time treatment could induce the transcription accumulation of CbγECS.

Similar to the cold treatment, other stresses, such as heat (42°C), NaCl and PEG6000, were all able to strongly induce the expression of CbγECS. In addition, as with AtγECS (Xiang and Oliver 1998) , CbγECS was induced by heavy metals, such as Cu^{2+} and Cd^{2+} (Fig. 6). These results reveal that CbγECS may be regulated by multiple environmental stresses. This induction in response to different stimuli has been reported for other molecules involved in plant defense responses, as pathogenesis-related genes, producing an overlapping response and cross-protection among environmental stresses (Chinnusamy et al. 2004).

In conclusion, a γ-ECS gene from *Chorispora bungeana*, which we denoted CbγECS, was identified and characterized. The expression of γ-ECS showed no tissue specificity and was induced by several environmental stresses, heavy metals, ABA and H_2O_2. The elucidation of γ-ECS regulation in *C. bungeana* requires the identification and molecular characterization of the individual components that participate in the regulation pathway in vivo. The results presented here represent an initial step toward the characterization and further understanding of the regulation of CbγECS under environmental stress conditions.

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References

An LZ, Liu YH, Feng GN, Feng HY, Chen T, Cheng GD (2000) Studies on ecological properties of altifrigetic subnival vegetation at the source area of Urumqi river. Acta Bot Boreali-Occident Sin 20:98–105

Ashida H, Sawa Y, Shibata H (2005) Cloning, biochemical and phylogenetic characterizations of gamma-glutamylcysteine synthetase from *Anabaena* sp. PCC 7120. Plant Cell Physiol 46:557–562

Ayitu R, Tan DY, Li ZJ, Yao F (1998) The relationship between the structures of vegetative organs in *Chorispora bungeana* and its environment. J Xinjiang Agric Univ 21:273–277

Chang JF, Fu XY, An LZ, Xu SJ, Wang JH, Zhang MX, Feng HY, Chen T (2006) Properties of cellular ubiquinone and stress-resistance in suspension-cultured cells of *Chorispora bungeana* during early chilling. Environ Exp Bot 57:116–122

Chinnusamy V, Schumaker K, Zhu J-K (2004) Molecular genetic perspectives on cross-talk and specificity in abiotic stress signalling in plants. J Exp Bot 55:225–236

Contour-Ansel D, Torres-Franklin ML, Cruz DECMH, D'Arcy-Lameta A, Zuily-Fodil Y (2006) Glutathione reductase in leaves of cowpea: cloning of two cDNAs, expression and enzymatic activity under progressive drought stress, desiccation and abscisic acid treatment. Ann Bot (Lond) 98:1279–1287

Copley SD, Dhillon JK (2002) Lateral gene transfer and parallel evolution in the history of glutathione biosynthesis genes. Genome Biol 3: research0025

Dixon DP, Lapthorn A, Edwards R (2002) Plant glutathione transferases. Genome Biol 3:reviews3004.1-3004.10

Dixon DP, Skipsey M, Grundy NM, Edwards R (2005) Stress-induced protein S-glutathionylation in *Arabidopsis*. Plant Physiol 138:2233–2244

Edwards R, Dixon DP, Walbot V (2000) Plant glutathione S-transferases: enzymes with multiple functions in sickness and in health. Trends Plant Sci 5:193–198

Foyer CH, Noctor G (2005) Redox homeostasis and antioxidant signaling: a metabolic interface between stress perception and physiological responses. Plant Cell 17:1866–1875

Fu XY, Chang JF, An LZ, Zhang MX, Xu SJ, Chen T, Liu YH, Xin H, Wang JH (2006) Association of the cold-hardiness of *Chorispora bungeana* with the distribution and accumulation of calcium in the cells and tissues. Environ Exp Bot 55:282–293

Gechev T, Gadjev I, Van Breusegem F, Inze D, Dukiandjiev S, Toneva V, Minkov I (2002) Hydrogen peroxide protects tobacco from oxidative stress by inducing a set of antioxidant enzymes. Cell Mol Life Sci 59:708–714

Gomez LD, Vanacker H, Buchner P, Noctor G, Foyer CH (2004) Intercellular distribution of glutathione synthesis in maize leaves and its response to short-term chilling. Plant Physiol 134:1662–1671

Guo FX, Zhang MX, Chen Y, Zhang WH, Xu SJ, Wang JH, An LZ (2006) Relation of several antioxidant enzymes to rapid freezing resistance in suspension cultured cells from alpine *Chorispora bungeana*. Cryobiology 52:241–250

Henmi K, Tsuboi S, Demura T, Fukuda H, Iwabuchi M, Ogawa KI (2001) A possible role of glutathione and glutathione disulfide in tracheary element differentiation in the cultured mesophyll cells of *Zinnia elegans*. Plant Cell Physiol 42:673–676

Hothorn M, Wachter A, Gromes R, Stuwe T, Rausch T, Scheffzek K (2006) Structural basis for the redox control of plant glutamate cysteine ligase. J Biol Chem 281:27557–27565

Ito H, Iwabuchi M, Ogawa K (2003) The sugar-metabolic enzymes aldolase and triose-phosphate isomerase are targets of glutathionylation in *Arabidopsis thaliana*: detection using biotinylated glutathione. Plant Cell Physiol 44:655–660

Janda T, Szalai G, Tari I, Paldi E (1999) Hydroponic treatment with salicylic acid decreases the effects of chilling injury in maize (*Zea mays* L.) plants. Planta 208:175–180

Jez JM, Cahoon RE, Chen S (2004) *Arabidopsis thaliana* glutamate-cysteine ligase: functional properties, kinetic mechanism, and regulation of activity. J Biol Chem 279:33463–33470

Kaminaka H, Morita S, Nakajima M, Masumura T, Tanaka K (1998) Gene cloning and expression of cytosolic glutathione reductase in rice (*Oryza sativa* L.). Plant Cell Physiol 39:1269–1280

Kocsy G, Brunner M, Ruegsegger A, Stamp P, Brunold C (1996) Glutathione synthesis in maize genotypes with different sensitivities to chilling. Planta 198:365–370

Kocsy G, Owttrim G, Brander K, Brunold C (1997) Effect of chilling on the diurnal rhythm of enzymes involved in protection against

oxidative stress in a chilling-tolerant and a chilling-sensitive maize genotype. Physiol Plant 99:249–254

Kocsy G, Szalai G, Vagujfalvi A, Stehli L, Orosz G, Galiba G (2000) Genetic study of glutathione accumulation during cold hardening in wheat. Planta 210:295–301

May MJ, Leaver CJ (1994) *Arabidopsis thaliana* gamma-glutamylcysteine synthetase is structurally unrelated to mammalian, yeast, and *Escherichia coli* homologs. Proc Natl Acad Sci USA 91:10059–10063

May MJ, Vernoux T, Leaver C, Van Montagu M, Inze D (1998a) Glutathione homeostasis in plants: implications for environmental sensing and plant development. J Exp Bot 49:649–667

May MJ, Vernoux T, Sanchez-Fernandez R, Van Montagu M, Inze D (1998b) Evidence for posttranscriptional activation of gamma-glutamylcysteine synthetase during plant stress responses. Proc Natl Acad Sci USA 95:12049–12054

Michelet L, Zaffagnini M, Marchand C, Collin V, Decottignies P, Tsan P, Lancelin JM, Trost P, Miginiac-Maslow M, Noctor G, Lemaire SD (2005) Glutathionylation of chloroplast thioredoxin f is a redox signaling mechanism in plants. Proc Natl Acad Sci USA 102:16478–16483

Mittova V, Theodoulou FL, Kiddle G, Gomez L, Volokita M, Tal M, Foyer CH, Guy M (2003) Coordinate induction of glutathione biosynthesis and glutathione-metabolizing enzymes is correlated with salt tolerance in tomato. FEBS Lett 554:417–421

Mora-Herrera ME, Lopez-Delgado H, Castillo-Morales A, Foyer CH (2005) Salicylic acid and H_2O_2 function by independent pathways in the induction of freezing tolerance in potato. Physiol Plant 125:430–440

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Neill S, Desikan R, Hancock J (2002a) Hydrogen peroxide signalling. Curr Opin Plant Biol 5:388–395

Neill SJ, Desikan R, Clarke A, Hurst RD, Hancock JT (2002b) Hydrogen peroxide and nitric oxide as signalling molecules in plants. J Exp Bot 53:1237–1247

Noctor G, Arisi A, Jouanin L, Kunert K, Rennenberg H, Foyer C (1998) Glutathione: biosynthesis, metabolism and relationship to stress tolerance explored in transformed plants. J Exp Bot 49:623–647

Noctor G, Gomez L, Vanacker H, Foyer CH (2002) Interactions between biosynthesis, compartmentation and transport in the control of glutathione homeostasis and signalling. J Exp Bot 53:1283–1304

Ogawa K (2005) Glutathione-associated regulation of plant growth and stress responses. Antioxid Redox Signal 7:973–981

Ogawa K, Tasaka Y, Mino M, Tanaka Y, Iwabuchi M (2001) Association of glutathione with flowering in *Arabidopsis thaliana*. Plant Cell Physiol 42:524–530

Ogawa K, Hatano-Iwasaki A, Yanagida M, Iwabuchi M (2004) Level of glutathione is regulated by ATP-dependent ligation of glutamate and cysteine through photosynthesis in *Arabidopsis thaliana*: mechanism of strong interaction of light intensity with flowering. Plant Cell Physiol 45:1–8

Ruiz JM, Blumwald E (2002) Salinity-induced glutathione synthesis in *Brassica napus*. Planta 214:965–969

Sanchez-Fernandez R, Fricker M, Corben LB, White NS, Sheard N, Leaver CJ, Van Montagu M, Inze D, May MJ (1997) Cell proliferation and hair tip growth in the *Arabidopsis* root are under mechanistically different forms of redox control. Proc Natl Acad Sci USA 94:2745–2750

Vandenabeele S, Van Der Kelen K, Dat J, Gadjev I, Boonefaes T, Morsa S, Rottiers P, Slooten L, Van Montagu M, Zabeau M, Inze D, Van Breusegem F (2003) A comprehensive analysis of hydrogen peroxide-induced gene expression in tobacco. Proc Natl Acad Sci USA 100:16113–16118

Vernoux T, Wilson RC, Seeley KA, Reichheld J-P, Muroy S, Brown S, Maughan SC, Cobbett CS, Van Montagu M, Inze D, May MJ, Sung ZR (2000) The root meristemless1/cadmium sensitive2 gene defines a glutathione-dependent pathway involved in initiation and maintenance of cell division during postembryonic root development. Plant Cell 12:97–110

Wachter A, Wolf S, Steininger H, Bogs J, Rausch T (2005) Differential targeting of GSH1 and GSH2 is achieved by multiple transcription initiation: implications for the compartmentation of glutathione biosynthesis in the Brassicaceae. Plant J 41:15–30

Wu J, Zhao Z, An L, Liu Y, Xu S, Gao D, Zhang Y (2008) Inhibition of glutathione synthesis decreases chilling tolerance in *Chorispora bungeana* callus. Cryobiology 57:9–17

Xiang C, Oliver DJ (1998) Glutathione metabolic genes coordinately respond to heavy metals and jasmonic acid in *Arabidopsis*. Plant Cell 10:1539–1550

Xiang C, Werner BL, Christensen EM, Oliver DJ (2001) The biological functions of glutathione revisited in arabidopsis transgenic plants with altered glutathione levels. Plant Physiol 126:564–574

Xiong L, Schumaker KS, Zhu J-K (2002) Cell signaling during cold, drought, and salt stress. Plant Cell 14:S165–S183

Zhang T, Liu Y, Xue L, Xu S, Chen T, Yang T, Zhang L, An L (2006) Molecular cloning and characterization of a novel MAP kinase gene in *Chorispora bungeana*. Plant Physiol Biochem 44:78–84