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BRIEF COMMUNICATION

Increased cold tolerance in *Arabidopsis thaliana* transformed with *Choristoneura fumiferana* glutathione *S*-transferase gene

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Abstract

A glutathione S-transferase (GST) gene cloned from the lepidopteran spruce budworm, *Choristoneura fumiferana* Clem. was transformed into the model plant *Arabidopsis thaliana*. The *CfGST*-transgenic and wild-type *A. thaliana* were subjected to 4 and 10 °C for 48 h and their cold resistance was studied. The GST activity of the transgenic plants was 46.6 and 35.7 % higher than that of the wild-type plants after 48 h under 4 and 10 °C, respectively. Relative membrane permeability and malondialdehyde content in the transgenic plants were lower while contents of the chlorophyll and proline were higher than those in the wild-type plants under 4 and 10 °C. The survival rate of the transgenic plants was 43.7 % for 24 h under 0 °C, while survival rate of wild-type plants was 28.3 %. The results indicated that the insect GST could enhance cold resistance in the transgenic *A. thaliana*.

Additional key words: chlorophyll, membrane permeability, malondialdehyde, proline, transgenic plants.

There has been increasing evidence showing that glutathione S-transferase (GST, E.C.2.5.1.18) is involved in stress responses in plants. Various external stresses, such as ozone, H_2O_2, heavy metals, herbicide, heat, dehydration, wounding, and toxic chemicals, have been reported to stimulate expression of *GST* genes in plants (Marrs 1996, Fujita and Hossain 2003, Hossain *et al.* 2006, Kosová *et al*. 2008). *GST* expression is influenced by stress-related hormones, including abscisic acid (ABA; Dixon *et al*. 1998), ethylene (Zhou and Goldsbrough 1993) and salicylic acid (SA; Wagner *et al*. 2002). Plant GSTs also play other roles, for example, catalyzing anthocyanins production (Marrs 1996, Sheehan *et al.* 2001). The role of GSTs in stress responses has been demonstrated in transgenic plants. For instance, over-expression of *GST* in tobacco (Roxas *et al*. 1997) and rice (Takesawa *et al*. 2002) enhanced the capability of the plants to endure high or low temperatures and high salt concentrations. Over-expression of *GST* can promote salt-resistance of transgenic tomato seedlings (Roxas 1997). *Suaeda salsa GST* gene was used to transform *A. thaliana* and over-expression of the gene increased *A. thaliana* growth in salt stress conditions (Qi *et al*. 2004). Oxidation of the reduced glutathione was suggested to be the mechanism for the increased salt resistance. Roles of GSTs in herbicide tolerance have also been demonstrated (Izuka *et al*. 1989, Irzyk and Fuerst 1993) and also in transgenic tobacco plants expressing a maize *GST* (Jepson *et al*. 1997).

The spruce budworm, *Choristoneura fumiferana* Clem, is one of the most widely distributed destructive forest insect pests in North America. This insect overwinters as a diapausing 2nd instar larva (Grisdale 1970). During this diapause Feng *et al.* (1999, 2001) found a high content of *Cf*GST protein, suggesting that this protein may play a role in its cold resistance. To test whether or not this insect GST can increase cold resistance in plants, we transformed *Arabidopsis thaliana* with the *CfGST* gene. In this paper we describe the generation of transgenic *A. thaliana* expressing *CfGST* gene and the responses of the transgenic plants to chilling stress.

The coding region (665 bp) of *CfGST* cDNA was obtained using polymerase chain reaction (PCR) and

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Abbreviations: CDNB-1 - chloro-2,4-dinitrobenzene; GST - glutathione S-transferase; MDA - malondialdehyde; RT-PCR - reverse transcription polymerase chain reaction.
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sequenced to confirm the absence of PCR-caused mutation. The PCR product was ligated into the pGTB binary vector between the sites of *Bam*H 1 and *Xba* I under the control of CaMV 35S promoter from pBI121. The linearized construct was then ligated to form a recombi- nant vector pGTB and the sequence was confirmed using PCR and sequencing. The pGTB vector was used to transform *Agrobacterium tumefaciens* LBA4404. A GST specific band of 665 bp was amplified by RT-PCR in kanamycin-ampicilin-resistance strains, indicating that the target *CfGST* gene was integrated into the genome of *A. tumefaciens* and expressed.

Wild-type *Arabidopsis* plants were transformed by floral dip method (Qi *et al*. 2004). T_1 seeds were collected and then transgenic plants were selected on MS medium supplemented with 1 % sucrose and 100 μg cm^{-3} kanamycin. Homozygous transgenic lines were identified in the T_2 generation. T_3 plants that were confirmed homzygous for the *CfGST* gene were grown and analyzed by duplex RT-PCR for the *CfGST* gene expression.

Arabidopsis thaliana L. ecotype Columbia (Col-0) was used as wild-type plants in this study. The T_2 seedlings of *CfGST*-transformed *Arabidopsis* plants were used for resistance analysis. Seeds were planted on Murashige and Skoog (1962 ;MS) agar plates or in a mixture of *Vermiculite*, peat moss and *Perlite* (1:1:1) for germination. Plants were grown in a growth chamber with 75 % relative humidity and a photoperiod of 16 h under white fluorescent tubes giving irradiance of 100 μmol m^{-2} s^{-1} and day/night temperature of 22/18 °C.

The wild type and *CfGST*-transgenic plants were maintained in the growth chamber for 4 weeks in the conditions described above. The chilling treatments were achieved by transferring the plants into chambers with temperature 10 and 4 °C, respectively, for 48 h. After chilling treatments, the plant samples were frozen in liquid nitrogen immediately and stored at -80 °C until analysis. The experiments were repeated at least three times.

Total RNA was extracted from the frozen samples using the modified phenol-chloroform method as described by Wan and Li (2005). The isolated RNA was treated with RNase-free DNase I (*TaKaRa*, Da Lian, China) at 37 °C for 1 h to eliminate DNA contamination. The *CfGST* gene expression was analyzed by duplex reverse transcription-PCR (RT-PCR) according to Sung *et al.* (2001). The concentration of cDNA produced by reverse transcription was estimated to be 100 μg cm^{-3}. The cDNA was then amplified with a pair of *CfGST*-gene specific primers and a pair of actin-specific primers in the same tube. A pair of *Arabidopsis* actin-specific primers and a pair of competitive primers were mixed at the ratio of 1:5 (actin primers: competitive primers) to generate duplex PCR signal. Twenty ng of cDNA was used for duplex PCR analysis in all of the experiments.

To investigate *CfGST* gene expression in the *Arabidopsis* plants in response to chilling stress, the GST gene-specific primers, forward primer 5'-TCTAGAATGGCC AAGAAACTACA-3' and reverse primer 5'-GGATCCTTATAGAGCATATGGTTT C-3') were used. The expected PCR product was 665 base pairs in length containing the complete coding region of the *Cf*GST gene. As an internal control, actin forward primer 5'-TATGTGGCTATTCAGGCTGT-3' and actin reverse primer 5'-TGGCGGTGCTTCTTCTCTG-3' were used to amplify a 750 bp fragment of the *Arabidopsis* actin gene. Duplex RT-PCR was conducted three times with three independently isolated total RNA samples. The gene specificity of RT-PCR was confirmed by sequencing all RT-PCR products.

After treatments leaves were cut into pieces and homogenized with a blender in 3 volumes of 50 mM Tris-HCl buffer (pH 7.5). The homogenate was filtered through six layers of gauze. The filtrate was centrifuged at 20 000 *g* for 30 min, and the resulting supernatant was used as the source of GST activity assay. GST activity was measured according to the standard procedures by employing 0.03 cm^3 aliquots of rapid translation system (RTS) solutions (Mannervik and Guthenberg 1981). GST activity toward the substrate 1-chloro-2,4-dinitrobenzene (CDNB) were measured in a total volume of 3 cm^3 RTS reaction containing 1 mM phosphate buffer, pH 6.5, 1 mM GSH and 0.05 cm^3 of enzyme preparation. The reaction was started by the addition of CDNB. The enzyme reaction was carried out at 30 °C for 30 min and enzyme activity was determined with spectrophotometer (*UV-2100, Shimadzu*, Japan) at 595 nm. Protein concentrations were measured using the *Bio-Rad* (Hercules, USA) protein reagent and bovine serum albumin as standard. Enzyme unit is mol(CDNB conjugated) min^{-1}.

The transgenic and wild type *A. thaliana* at 6-leaves seedling stage were placed in a controlled environmental chamber under irradiance of 80 μmol m^{-2} s^{-1} and at 0 °C for 24 h. The plants were then moved to the normal growth chamber (conditions as described above) for 2 d. Survival rates of the plants were calculated as the ratio of alive seedlings with green leaves to all seedlings used. The relative membrane permeability was determined as described by Li and Pan (1996). Malondialdehyde (MDA) content was assayed using the thiobarbituric acid reaction method according to Draper and Hadley (1990). The chlorophyll content was determined in 80 % (v/v) acetone extract according to MacKinney (1941). The absorption was measured with a spectrophotometer (*UV-2100*) at room temperature. Proline content was determined using the molybdenum blue method (Bates *et al*. 1973). The absorbance was detected at 650 nm.

Ninety five *A. thaliana* plants were transformed with recombinant *Agrobacterium* LBA4404 harboring the *CfGST* gene, producing 15 000 seeds. These seeds were germinated on 1/2 MS medium containing kanamycin. All non-transgenic seedlings were etiolated after germination and then died after 10 d on the medium. Six kanamycin-resistance T_2 lines were obtained and confirmed for the presence and expression of the *CfGST* gene using PCR. A 665-bp *CfGST*-specific band, which was confirmed by sequencing, was detected when genomic DNA was used as

template (Fig. 1*A*). Wild-type plants did not have this specific band. This result indicated that the *CfGST* gene had been integrated into the genome of the transgenic *A. thaliana* plants. To further study whether or not the inserted *CfGST* expressed in the transgenic plants, expression of *CfGST* gene was examined using RT-PCR. A 665-bp PCR product was detected in the transgenic plants, whereas no such a band was detected in the wild-type plants, indicating that *CfGST* gene was not only integrated into the genome but also expressed in these six transgenic lines (Fig. 1*B*). The plants of the six *CfGST*-transgenic lines were dark green and had strong root systems consisting of axial and lateral roots.

It was difficult, if not impossible, to distinguish the GST induced by inserted *CfGST* from that present constitutively. Therefore, only total GST activity was tested in both the wild-type and the transgenic plants after they were transferred from 23 to 4 or 10 °C (Fig. 2). At 23 °C, the transgenic plants had a higher GST activity than the wild-type plants. However, after the plants were transferred to the low temperatures, the GST activity in the transgenic plants increased. After 48 h at 4 and 10 °C the GST activity in the transgenic plants was 46.6 and 35.7 %, respectively, higher than that in the wild-type plants.

Relative membrane permeability, usually presented as electric conductivity ratio, is an important indicator of cold damage in plants. When the plants were transferred from 23 to 4 or 10 °C, membrane permeability in both the

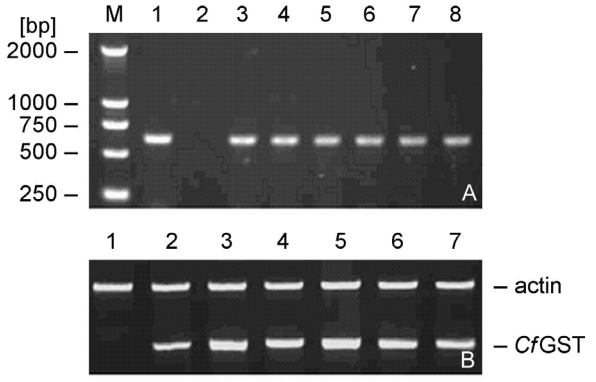

Fig. 1. Detection of *CfGST* gene insertion in the genomes of the *CfGST*-transformed *A. thaliana* T_3 lines (*A*) and mRNA expression of *CfGST* (*B*). *A*: M - DNA marker, *lane* 1 - positive control, the plasmid containing CfGST gene, *lane* 2 - wild-type plant, *lanes* 3 to 8 - six transgenic plants (six different lines). *B*: *lane* 1 - wild-type plant, *lanes* 2 to 7 - six transgenic plants. The CfGST product is 665 bp. Actin was used as an internal indicator of equal loading of mRNA.

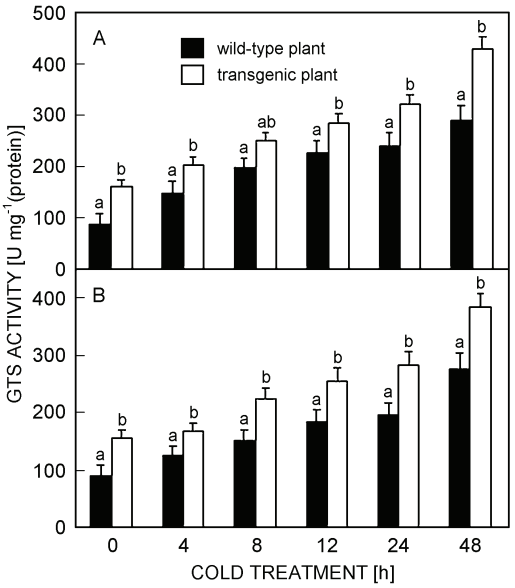

Fig. 2. GST activity in leaves of the T_2 *CfGST*-transgenic and wild-type *A. thaliana* seedlings at 4 °C (*A*) and 10 °C (*B*). Means ± SE of 4 leaves. Different letters denote significant differences at $P \leq 0.05$.

Table 1. Relative membrane permeability, MDA content, chlorophyll content and proline content in leaves of the *CfGST*-transgenic and wild-type *A. thaliana* seedlings treated at 4 and 10 °C upto 48 h. Data are means ± SD of 4 leaves, different letters denote significant differences at $P < 0.05$, nd - not detected.

[°C]	Time [h]	Membrane permeability [%]		MDA [μmol g^{-1}(f.m.)]		Chlorophyll [mg g^{-1}(f.m.)]		Proline [μg g^{-1}(f.m.)]	
		wild type	transgenic	wild type	transgenic	wild type	transgenic	wild type	transgenic
4	0	nd	nd	3.09 ± 0.3a	2.93 ± 0.2b	21.2 ± 1.0a	20.5 ± 0.6a	100 ± 10a	89 ± 15a
	4	54.3 ± 1.3a	35.2 ± 1.8b	8.17 ± 0.4a	5.11 ± 0.2b	18.5 ± 0.8a	18.0 ± 1.0a	860 ± 20a	890 ± 20a
	8	63.2 ± 1.5a	43.1 ± 2.5b	7.33 ± 0.2a	3.78 ± 0.1b	17.4 ± 1.4a	17.6 ± 0.5a	920 ± 25a	990 ± 20a
	12	64.3 ± 2.5a	50.2 ± 2.8b	8.91 ± 0.2a	4.22 ± 0.3b	15.3 ± 1.3a	16.6 ± 1.0ab	1211 ± 28a	1325 ± 10ab
	24	70.2 ± 2.5a	60.2 ± 2.3ab	9.38 ± 0.3a	5.33 ± 0.2b	14.4 ± 2.0a	15.4 ± 0.9a	1475 ± 31a	1517 ± 12a
	48	79.2 ± 3.5a	61.1 ± 1.8b	10.20 ± 0.1a	5.56 ± 0.1b	12.8 ± 1.4a	14.4 ± 0.8b	1596 ± 31a	1775 ± 20b
10	0	nd	nd	3.09 ± 0.3a	2.93 ± 0.2a	22.0 ± 1.2a	21.6 ± 0.9a	100 ± 10a	89 ± 15a
	4	42.5±1.8a	31.2 ± 2.5b	3.20 ± 0.3a	3.02 ± 0.2a	20.3 ± 1.0a	21.0 ± 1.0a	175 ± 11a	185 ± 13a
	8	43.6±2.3a	40.1 ± 3.2ab	3.56 ± 0.2a	3.38 ± 0.1a	18.6 ± 1.4a	19.5 ± 0.7ab	250 ± 15a	245 ± 15a
	12	57.2±3.2a	55.2 ± 3.0a	4.89 ± 0.2a	3.93 ± 0.2b	17.4 ± 1.2a	18.9 ± 1.0b	301 ± 25a	383 ± 18b
	24	65.2±2.5a	48.0 ± 2.8b	5.60 ± 0.3a	4.03 ± 0.1b	16.8 ± 1.9a	18.0 ± 0.9ab	397 ± 26a	390 ± 10a
	48	64.1±3.8a	48.5 ± 2.5b	6.00 ± 0.1a	4.27 ± 0.1b	16.4 ±1.3a	17.6 ±1.3b	415 ± 25a	517 ± 16b

wild-type and the transgenic plants increased with the time in cold treatments. The 4 °C treatment resulted in a higher membrane permeability than the 10 °C treatment and the transgenic plants had always lower membrane permeability than the wild-type plants (Table 1). The survival rate of the transgenic plants after treatment at 0 °C for 24 h was 43.7 %, while survival rate of the wild-type plants was only 28.3 %.

Malondialdehyde (MDA) content of plant leaves is indicator of oxidative stress and membrane damage resulted from cold stress. MDA contents increased after the wild-type and transgenic plants were transferred to low temperatures (Table 1). At 10 °C there was no significant difference in MDA content between the wild-type plants and the transgenic plants before 12 h in the cold treatment, but lower MDA content was found in the transgenic plants than in the wild-type plants after longer cold treatment. At 4 °C for 48 h, the MDA content in the transgenic plants was 45.5% lower than that in the wild-type plants.

Chlorophyll content is susceptible to cold stress and usually continuously declines with cold stress (Gajewska *et al*. 2006). In this study, chlorophyll content in the wild-type and the transgenic plants decreased after 4 and 10 °C treatment. However, although the transgenic plants appeared to have higher chlorophyll content than the wild-type plants, the difference was not significant before 24 h of cold stress (Table 1).

Proline is a substance for protecting membrane permeability and maintaining the osmotic balance of cells during cold stress. No significant difference in proline content was detected between the transgenic and wild-type plants before 24 h in the cold treatments (Table 1). However, the proline content in the transgenic plants was 11.2 and 24.6 % higher than in the wild-type plants at 4 and 10 °C, respectively, after 48-h treatments.

All of the above results together indicated that the *CfGST*-transgenic *A. thaliana* plants gained higher resistance against the cold stress than the wild-type plants, as reflected by increase in survival rate, chlorophyll and proline contents and decrease in membrane permeability and MDA content.

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