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Molecular characterization and expression of three galactinol synthase genes that confer stress tolerance in *Salvia miltiorrhiza*

Donghao Wang, Wei Yao, Yin Song, Wenchao Liu, Zhezhi Wang*

Key Laboratory of the Ministry of Education for Medicinal Resources and Natural Pharmaceutical Chemistry, National Engineering Laboratory for Resource Development of Endangered Crude Drugs in Northwest of China, College of Life Sciences, Shaanxi Normal University, Xi'an 710062, PR China

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ABSTRACT

To adapt to changes in their growing environment, plants express several stress-responsive genes. For example, the products of *galactinol synthase* (*Gols*) genes play a key role in regulating the levels of raffinose family oligosaccharides and conferring resistance to stress. We cloned and characterized three *Gols* genes in *Salvia miltiorrhiza*. Their expression followed three distinct patterns. Compared with the control, *SmGols1* was up-regulated by temperature changes but was suppressed by exposure to methyl jasmonate or short-term drought. This gene had the greatest abundance of transcripts and was assigned a general function of carbon storage. *SmGols2* responded to all stress and hormone treatments, and transcripts were maintained at a high level. Finally, expression of *SmGols3* was weaker than the other two genes, but was increased significantly under different treatments. Over the experimental period, its expression declined to normal levels in response to all treatments except exposure to 100 μM ABA, long-term drought, heat (42 °C), or chilling (8 °C). Based on our finding of *cis*-elements in the 5′ flanking regions, we concluded that these genes seem to be regulated by several *HSF* transcription factors. We also targeted their 90-bp conserved sequences and used them for RNA interference analysis. Some were knocked down to various extents in our transgenic lines. Fluctuations in their malondialdehyde contents under different stress treatments, as well as the rate of water loss in transformed plants, suggested that lipid peroxidation was more likely to occur in the transgenics than in the control. These results indicate that *SmGols* genes could have a main function in responding to cold or heat. Therefore, we believe that it is important to investigate this mechanism for tolerance in *S. miltiorrhiza* and to examine how expression of these *SmGols* and other homologs are influenced by abiotic stresses.

Introduction

Plants invoke common mechanisms in their physiological responses to changing environmental conditions (Shinozaki et al., 2003; Fujita et al., 2006; Yamaguchi-Shinozaki and Shinozaki, 2006). Over time, periods of drought, high salinity, and extremes in temperature have become more frequent. A better understanding of the stress response pathways in plants improve our investigations of those mechanisms at the genetic and physiological levels so that we can better utilize these species as important sources of valuable ingredients for health and nutrition. In response to environmental pressures on plant cells, the products of many genes function in the accumulation of regulatory components for osmosis, transcriptional regulation, and protection against the effects of osmotic, cold, and abiotic stresses. These components include mannitol, proline, and a large number of soluble oligosaccharides.

Raffinose family oligosaccharides (RFOs), e.g., raffinose, stachyose, and verbacose, are soluble galactosyl-sucrose carbohydrates with several roles in plant development. Their accumulation is often associated with stressful environmental conditions, including cold, heat, or dehydration (Peterbauer et al., 2001; Downie et al., 2003; Peters et al., 2007). Galactinol synthase (Gols; EC2.4.1.123) catalyzes synthesis of galactinol from UDP-galactose and myo-inositol. Galactinol is a exclusively galactosyl donor for raffinose family oligosaccharides biosynthetic pathway, thus Gols plays an important regulatory role in carbon-partitioning between sucrose and RFOs (Saravitz et al., 1987). Therefore, because Gols is potentially a metabolic control point, understanding its associated gene would allow us to manipulate the levels of RFOs and enhance abiotic stress tolerance in plants.

Galactinol synthase is a monomeric protein that is activated in a pH range of 7.0–8.0. This activity can be further enhanced by treatment with DDT (dithiothreitol) and Mn^{2+} (Handley et al., 1983;

Abbreviations: ABA, abscisic acid; CTAB, hexadecyl trimethyl ammonium bromide; DDT, dithiothreitol; Gols, galactinol synthase; MDA, malondialdehyde; MeJA, methyl jasmonate; NaCl, sodium chloride; RFO, raffinose family oligosaccharide; RNAi, RNA interference; SA, salicylate.

* Corresponding author. Tel.: +86 29 85310260; fax: +86 29 85310546.
E-mail address: zzwang@snnu.edu.cn (Z. Wang).

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Pharr et al., 1985; Smith et al., 1991). While the exogenous application of abscisic acid (ABA) induces a several-fold accumulation of galactinol and RFOs in alfalfa (*Medicago sativa* L.) or tiny vetch (*Vicia hirsuta* [L.] S.F. Gray) (Lahuta et al., 2004; Blöchl et al., 2005), Gols activity is also increased by three-fold over the control in somatic embryos of alfalfa (Blöchl et al., 2005). Although investigations have been conducted on the role of Gols in seed development, little is known about its functioning in plant vegetative organs. Several previous studies have shown that the *CmGols1* (*Cucumis melo*) promoter is activated in the smallest veins of mature leaves from tobacco and *Arabidopsis*, suggesting that the expression pattern of Gols is consistent with a role in sugar synthesis and phloem loading in cucurbit species as well. Two *Gols* genes (*VpGols1* and *VpGols2*) have been cloned and expressed in the leaf intermediary cells of *Verbascum phoeniceum* L.; results from their RNAi suppression have indicated that the synthesis of RFOs in those cells is necessary for efficient phloem transport, a conclusion consistent with the polymer trap model of phloem loading (McCaskill and Turgeon, 2007). Similarly, results from an examination of two *PaGols* genes in hybrid poplar have implied that *PaGols1* is involved in basic metabolic activities, such as carbon storage, and that *PaGols2* probably participates in the seasonal mobilization of carbohydrates (Unda et al., 2012). Using *Ajuga*, Sprenger and Keller (2000) have reported that *AjGols1* is involved in the storage of oligosaccharides while *AjGols2* acts in their transport. Under low-temperature stress, oligosaccharide contents increase in both mesophyll cells and phloem cells. Seven *Gols* genes have been identified in *Arabidopsis*. They include *AtGols1* and *AtGols2*, which are induced by drought and high salt, respectively, and *AtGols3*, which responds to chilling (Taji et al., 2002). In addition, over expression of *AtGols2* in *Arabidopsis* causes an increase in the levels of galactinol and RFOs, as well as enhances leaves dehydration tolerance (Taji et al., 2002). Three *Gols* genes have been cloned and analyzed in *Coffea arabica* L. Expression of *CaGols2* is detected only under severe water deficits and salt stress, *CaGols3* functions under drought conditions, and the *CaGols1* isoform is responsive to all tested stress treatments. The accumulation of RFOs in *C. arabica* leaves corresponds to an increase in transcriptional activity, indicating that *CaGols* have an important function in protection against osmotic stresses (dos Santos et al., 2011).

Stress-related Gols genes have also been found in other species. For example, under low temperatures, transcripts are highly expressed in the vegetative tissues of red kidney bean (*Phaseolus vulgaris* var. Big Red) (Liu et al., 1998); the level of *OsGols1* mRNA was increased associated with raffinose accumulation in rice seedlings (Saito and Yoshida, 2011). The genome for maize (*Zea mays*) contains *ZmGols2*, induced primarily by dehydration, and *ZmGols3*, which accumulates when germinating seeds are heat-stressed (Zhao et al., 2003, 2004). In germinating seeds of tomato (*Lycopersicon esculentum*), the accumulation of *LeGols1* mRNA is stimulated by dehydration but not by cold, whereas both stresses induce this accumulation in seedling leaves, thereby suggesting that *LeGols1* is indirectly involved in conferring desiccation tolerance in that species (Downie et al., 2003), while the same conclusion was found that *BnGols1* transcripts was relatively high associated with desiccation tolerance and complicated the further accumulation of RFOs during *Brassica napus* seeds development (Li et al., 2011). Finally, Takanashi et al. (2008) have used a special cDNA library to isolate a gene encoding *CjGols* in *Coptis japonica*. Although the exact mechanism is still unknown, researchers have confirmed that this gene is involved in providing resistance to the influence of berberine in that species.

Salvia miltiorrhiza Bunge (Danshen) is a well-known traditional Chinese medicine that is widely used for the treatment of cardiovascular and cerebrovascular diseases. Commonly found throughout China and other Southeast Asian countries, this herb contains numerous stress-inducible genes that enable it to adapt to various biological environments and sources of stress. As a medicinal plant, it also produces many valuable metabolites. Being a key enzyme of a primary metabolite, Gols is not only closely related to sugar, but its products play an important role in stress tolerance. Here, we isolated three *SmGols* genes and monitored their differential transcriptional activities. We also utilized RNAi suppression to examine physiological indicators in transgenic plants as a means of studying the functioning and mechanisms for stress tolerance by these genes in *S. miltiorrhiza*.

Materials and methods

Plant materials, growing conditions, and stress treatments

Seeds of *Salvia miltiorrhiza* Bunge were germinated in sterilized vermiculite, where they were irrigated with water and a 1/2-strength MS mineral nutrient solution (Murashige and Skoog, 1962). After the fourth true leaf appeared, the seedlings were transferred to 500-mL pots containing sterilized vermiculite, then moved to a greenhouse where they were watered at 2-day intervals. Whole seedlings were used for DNA extraction. Prior to our stress treatments, the plants were acclimated for one week in a growth chamber set at $25 \pm 2\,^{\circ}\mathrm{C}$, and under a 16-h photoperiod provided by cool white fluorescent lamps ($25\,\mu\mathrm{mol\,m^{-2}\,s^{-1}}$) (Song and Wang, 2008).

Short-term drought was induced in the pots by replacing the aquiferous vermiculite with dry filter paper. Samples were collected after 0, 10, 30, 60, 80, 100, and 120 min. For long-term drought, seedlings were irrigated firstly and then were kept without watering. Samples were collected after 0, 10, or 20 d. Samples were taken at each time point. To check their response to temperature extremes, seedlings were exposed to either 8 °C (cold) or 42 °C (heat). Other plants were treated with 200 mM NaCl, 100 μM ABA, 5 mM methyl jasmonate (MeJA), or 3 mM salicylate (SA). When the plants were soaked in a final concentration of 200 mM NaCl, 100 μM ABA, 5 mM methyl jasmonate (MeJA), or 3 mM SA, the three hormones (ABA, MeJA, SA) were sprayed for leaves respectively at the same time. Samples were then collected after 0, 1, 3, 6, 12, 24, or 48 h of such treatments. All tissues were immediately frozen in liquid nitrogen and stored at −80 °C prior to RNA isolation.

Nucleic acid extraction

Genomic DNA was extracted from whole seedlings by the modified CTAB method (Doyle and Doyle, 1987). Total RNA was isolated from frozen samples, using Plus reagent (Takara) as recommended by the manufacturer. The quality and concentrations of RNA and genomic DNA were determined by 1.0% agarose gel electrophoresis and analysis with a spectrophotometer (SHIMADZU UV-2450). The cDNA was obtained through a process in total 20 μL involving an oligo $(\mathrm{dT})^{18}$ primer (0.05 μmol), reverse transcriptase (200 U), a ribonuclease inhibitor (20 U), dNTPs (Takara) (0.04 μmol), some diethylpyrocarbonate (DEPC)-treated water and isolated RNA (1 μg) according to the manufacturer's instructions.

RT-PCR amplification of cDNA and genome sequencing

After screening the cDNA library for *S. miltiorrhiza* that was constructed and conserved by our laboratory (Yan et al., 2010), we obtained an 1139-bp EST fragment (GenBank Accession Number CV167095). Alignment revealed that this sequence lacked the N-terminal and was named as *SmGols1*. Using total DNA and cDNA as templates, the DNA and cDNA partial sequences of *SmGols1* were obtained via PCR with Primers Gols1Fb/Gols1Rb (Table 1). Conditions for two-step PCR included one cycle of pre-denaturation at 94 °C for 5 min; then 40 cycles of 94 °C for 30 s, and annealing and