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Important roles of drought- and cold-inducible genes for galactinol synthase in stress tolerance in *Arabidopsis thaliana*

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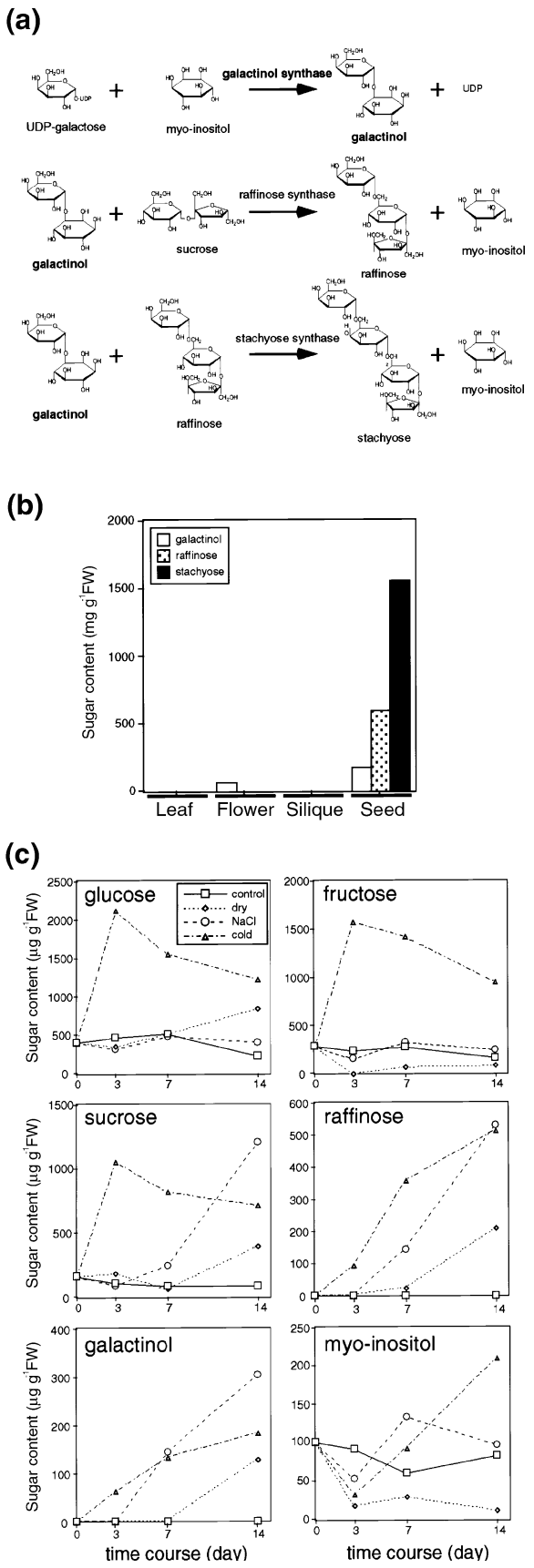
Summary

Raffinose family oligosaccharides (RFO) accumulating during seed development are thought to play a role in the desiccation tolerance of seeds. However, the functions of RFO in desiccation tolerance have not been elucidated. Here we examine the functions of RFO in *Arabidopsis thaliana* plants under drought- and cold-stress conditions, based on the analyses of function and expression of genes involved in RFO biosynthesis. Sugar analysis showed that drought-, high salinity- and cold-treated *Arabidopsis* plants accumulate a large amount of raffinose and galactinol, but not stachyose. Raffinose and galactinol were not detected in unstressed plants. This suggests that raffinose and galactinol are involved in tolerance to drought, high salinity and cold stresses. Galactinol synthase (GolS) catalyses the first step in the biosynthesis of RFO from UDP-galactose. We identified three stress-responsive *GolS* genes (*AtGolS1, 2* and *3*) among seven *Arabidopsis GolS* genes. *AtGolS1* and *2* were induced by drought and high-salinity stresses, but not by cold stress. By contrast, *AtGolS3* was induced by cold stress but not by drought or salt stress. All the GST fusion proteins of GST-AtGolS1, 2 and 3 expressed in *Escherichia coli* had galactinol synthase activities. Overexpression of *AtGolS2* in transgenic *Arabidopsis* caused an increase in endogenous galactinol and raffinose, and showed reduced transpiration from leaves to improve drought tolerance. These results show that stress-inducible galactinol synthase plays a key role in the accumulation of galactinol and raffinose under abiotic stress conditions, and that galactinol and raffinose may function as osmoprotectants in drought-stress tolerance of plants.

Keywords: drought tolerance, transgenic plants, galactinol synthase (GolS), RFO, galactinol, raffinose.

Introduction

Plants respond to water deficit and adapt to drought conditions through various physiological and biochemical changes, including transition in gene expression during water deficit. The mechanisms of drought-stress response have been investigated most extensively in a model plant, *Arabidopsis thaliana*, and in other plants including crops and resurrection plants (Bray, 1997; Ingram and Bartels, 1996; Shinozaki and Yamaguchi-Shinozaki, 1997; Shinozaki and Yamaguchi-Shinozaki, 1999). The drought-stress signal is mediated through both abscisic acid (ABA)-dependent and ABA-independent pathways to regulate expression of various genes that are involved in drought-stress tolerance and response. These gene products are thought to function in the accumulation of osmoprotectants; detoxification protection of cells; protein turnover; stress-signalling pathways; transcriptional regulation, and so on (Bohnert *et al.*, 1995; Bray, 1997; Ingram and Bartels, 1996; Shinozaki and Yamaguchi-Shinozaki, 1997; Shinozaki and Yamaguchi-Shinozaki, 1999). In particular, osmoprotectants such as proline, glycinebetaine and mannitol have

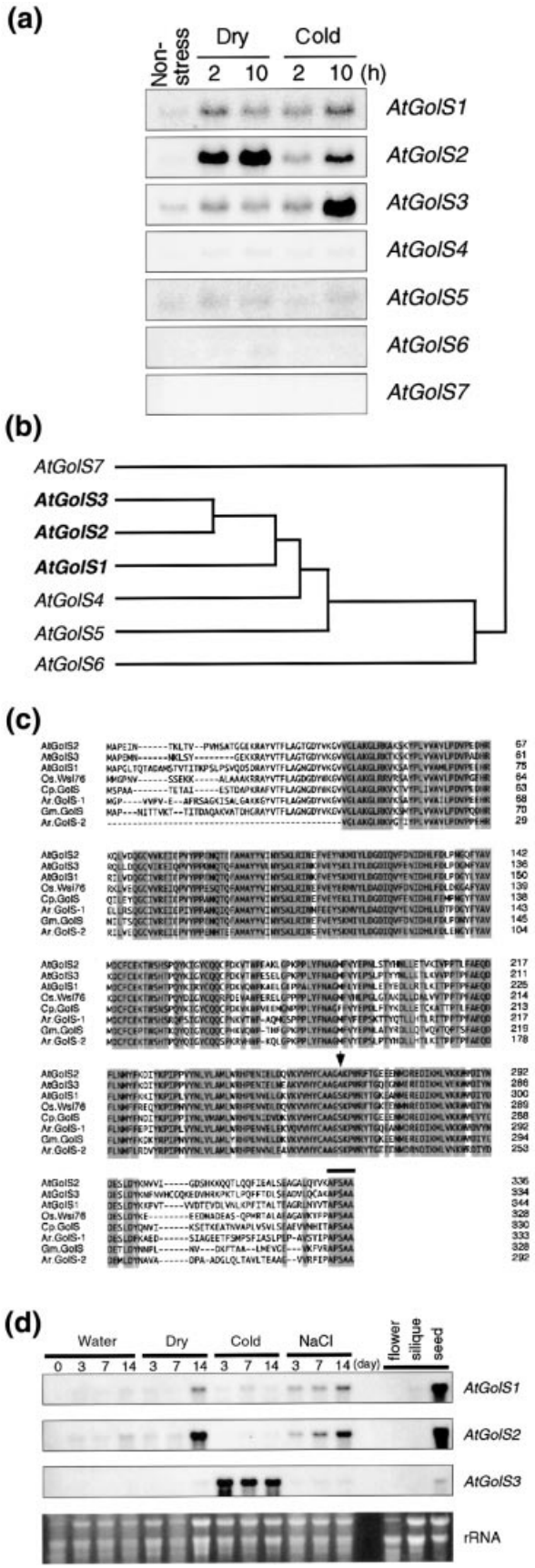


been shown to be important for the improvement of stress tolerance in plants by manipulation of genes encoding key enzymes of the osmolyte synthesis or degradation pathway (Kavi-Kishor *et al.*, 1995; Nanjo *et al.*, 1999a; Nanjo *et al.*, 1999b; Tarczynski *et al.*, 1993; Thomas *et al.*, 1995). Raffinose family oligosaccharides (RFOs) such as raffinose and stachyose accumulate during seed development and are thought to play some role in desiccation tolerance of seeds. During seed development, RFOs accumulate at the late stage of soyabean seed maturation and desiccation (Castillo *et al.*, 1990; Saravitz *et al.*, 1987). In maize, raffinose accumulates during the seed desiccation process and is thought to function in stress tolerance, whereas sucrose accumulates independently of desiccation tolerance. Desiccation tolerance of seeds is not achieved in the absence of raffinose accumulation (Brenac *et al.*, 1997). These results suggest that the ratio of sucrose to RFO is critical for desiccation tolerance of seeds, rather than the total amount of sugars. Although young, excised soyabean seeds are not tolerant to desiccation, slow dehydration induces stress tolerance, which is strongly correlated with a significant increase in stachyose content (Blackman *et al.*, 1992).

Figure 1(a) shows the metabolic pathway of RFOs in plants. Galactinol synthase (GolS) catalyses the first committed step in the biosynthesis of RFOs and plays a key regulatory role in carbon partitioning between sucrose and RFOs (Saravitz *et al.*, 1987). Therefore GolS potentially catalyses a metabolic key step, and its gene provides an experimental tool to manipulate the level of RFOs in seeds or vegetative tissues to analyse the function of RFOs as osmoprotectants. The GolS activity in kidney bean seeds increases on exposure of plants to cold, and the expression of *GolS* genes is induced by cold stress in *Arabidopsis* and *Ajuga reptans* plants (Liu, J. J. *et al.*, 1998; Sprenger and Keller, 2000). During cold acclimation, RFOs accumulate in leaves of *A. reptans* (Bachmann *et al.*, 1994). However, functions of RFOs in stress tolerance, and roles of GolS in RFO biosynthesis, have not been elucidated.

At least seven *GolS*-related genes are found in the *Arabidopsis* genome, but little is known about their roles in the accumulation of galactinol and raffinose in plants

Figure 1. Carbohydrate content of various tissues and whole *Arabidopsis* plants under water stress.
(a) Metabolic pathway of galactinol and RFO (raffinose and stachyose) in plants.
(b) RFO and galactinol contents of leaf, flower, silique and seed in *Arabidopsis*.
(c) Effect of drought, high salinity and cold stresses on carbohydrate content of vegetative tissues of *Arabidopsis*.
Three-week-old soil-grown plants were exposed to drought (dry, ◇); high salinity (150 mM NaCl, ○); or cold (4°C, △) stresses, and untreated (control, □). Stress treatment is described under Experimental procedures.



under water-deficit conditions. In the present study we report that three *Arabidopsis GolS* genes, *AtGolS1*, *2* and *3*, are differentially induced by drought, low temperature, high salinity and ABA. We showed that their gene products have galactinol synthase activity. Furthermore, transgenic plants that overexpressed the *AtGolS* cDNA showed improved drought tolerance. This study provides a direct evidence that the stress-inducible *GolS* gene controls the level of RFOs, and that galactinol and raffinose play important roles in drought-stress tolerance.

Results

Accumulation of galactinol and RFOs during water-deficit stress in Arabidopsis

We measured the accumulation of endogenous levels of galactinol and RFOs in *Arabidopsis* reproductive organs or soil-grown *Arabidopsis* plants during dehydration, high salinity and cold stresses. As shown in Figure 1(b), galactinol, raffinose and stachyose accumulated only in seeds in reproductive organs. Sucrose is the first major sugar in *Arabidopsis* mature seeds, and stachyose and raffinose are the second major sugars. This suggests that galactinol, raffinose and stachyose act as osmoprotectants in *Arabidopsis* seeds as in other plants such as soyabean (*Glycine max*) (Blackman *et al.*, 1992). So the sugar content was also measured in *Arabidopsis* plants that had been treated with dehydration, high salinity (150 mM NaCl) or cold stress, and in unstressed plants (water) as a control (Figure 1c). While the unstressed plants had no detectable amount of RFOs, galactinol and raffinose accumulated in all the stress-treated plants. *O*-methyl-inositol, which functions in *Coleus* as

Figure 2. Northern analysis of seven galactinol synthase genes and comparison of their deduced amino acid sequences.
(a) Northern analysis of seven *AtGolS* genes under drought and cold stress. Each lane was loaded with 20 μg total RNA prepared from wild-type plants that had been exposed to drought (dry) or cold (4°C) stress for 2 or 10 h. The membranes were hybridized with [^{32}P]-labelled *AtGolS* cDNAs as probes.
(b) The phylogenetic tree of *AtGolS* genes.
(c) Comparison of deduced amino acid sequences from cDNAs encoding galactinol synthase. Compared galactinol synthases are three of *Arabidopsis thaliana* (AtGolS1, 2, 3), *Ajuga reptans* (Ar.GolS-1, 2; Sprenger and Keller, 2000), *Glycine max* (Gm.GolS; Kerr *et al.*, 1993), *Cucurbita pepo* (Cp.GolS; Kerr *et al.*, 1993) and *Oryza sativa* (Os.Wsi76; Takahashi *et al.*, 1994). The putative serine phosphorylation site is shown by an arrow. A characteristic hydrophobic pentapeptide (APSAA) is shown as a bar.
(d) Northern blot analysis of the induction of the *AtGolS* genes by water-deficit stresses and in various organs. Total RNA was isolated from various *Arabidopsis* organs and whole plants which were treated in the same ways as those in Figure 1(b,c). Each lane was loaded with 5 μg total RNA. The RNA was fractionated on a 1% agarose gel, blotted onto a nylon membrane, and probed with digoxigenine; DIG-labelled cDNA inserts of the *AtGolS* cDNAs.

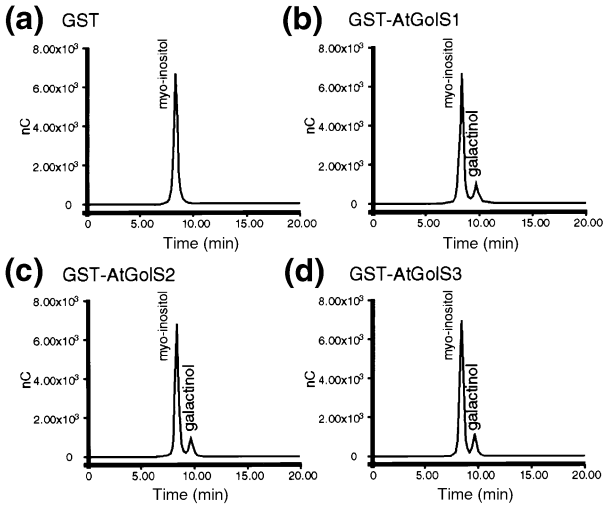


Figure 3. HPLC profiles of carbohydrate metabolites.
Profiles of carbohydrate metabolites of GST (a), GST-AtGolS1 (b), GST-AtGolS2 (c), and GST-AtGolS3 (d)recombinant proteins. The reaction mixture contained myoinositol as substrate.

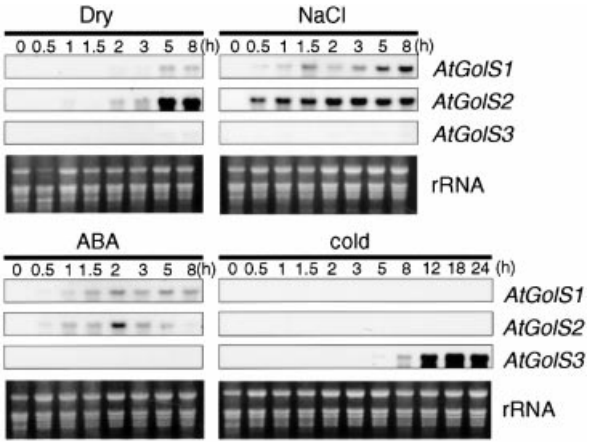


Figure 4. Northern analysis of induction of the *AtGolS* genes by dehydration, high-salinity and cold stresses, and ABA treatment.
Total RNA was prepared from 3-week-old *Arabidopsis* plants grown on GM plates with or without stress treatment. Stress treatment was given as follows: transfer plants from agar plates on Whatman 3MM filter paper (dry); transfer plants from agar plates to hydroponic culture with 250 mM NaCl (NaCl); transfer plants from agar plates to hydroponic culture with 100 μM ABA (ABA); transfer plants on agar plates to growth chamber at 4°C (cold). Each lane was loaded with 5 μg total RNA. The RNA was fractionated on a 1% agarose gel, blotted onto a nylon membrane, and hybridized with digoxigenine; DIG-labelled cDNA inserts of the *AtGolS* cDNAs as probes.

an osmoprotectant, was not detected under these stresses in *Arabidopsis* (Pattanagul and Madore, 1999). These results imply that galactinol and raffinose function as an osmoprotectant in plants under osmotic stresses. However, stachyose, the first major RFO in seeds, did not accumulate in these stressed plants.

Isolation and characterization of GolS *genes from* Arabidopsis

Seven *GolS*-related genes were identified from the *A. thaliana* (Columbia) genome databases (AtDB, Stanford University; http://genome-http://www.stanford.edu/Arabidopsis/) by a homology search against the *Oryza sativa GolS* gene homologue, *wsi76* (Takahashi *et al.*, 1994), using the BLAST program. Their GenBank accession numbers are AC002337, AC009323, AC003970, AC002292, AB005244, AL049171AL161564and AC004473. We named these genes *AtGolS1*, *2*, *3*, *4*, *5*, *6* and *7*, respectively. The putative *GolS* genes were amplified from the *Arabidopsis* full-length cDNA library (Seki *et al.*, 1998) by PCR using the synthetic oligonucleotide sets (see Experimental procedures for details), and into the pBluescript vector. To investigate the stress-inducibility and tissue-specific expression of the seven *AtGolS* genes, we carried out the Northern blot analysis using *Arabidopsis* plants grown on agar plates (Figure 2a). Among seven *AtGolS* genes, three genes were induced by osmotic stress. These cDNAs were isolated by screening the *Arabidopsis* full-length cDNA library using the above-mentioned PCR-amplified cDNA fragments as probes and named *AtGolS1*, *2* and *3*, respectively. Phylogenic analysis revealed that *AtGolS1*, *2* and *3* are highly homologous with each other (Figure 2c), and are all highly homologous to known *GolS* genes from *A. reptans* (Sprenger and Keller, 2000); soyabean (Kerr *et al.*, 1993); zucchini (Kerr *et al.*, 1993); and rice (Takahashi *et al.*, 1994). All of the reported GolS and AtGolS1 proteins have a putative serine phosphorylation site at position 270, but AtGolS2 and 3 do not. There is a characteristic hydrophobic pentapeptide (APSAA) at the carboxy-terminal end of all known *GolS*.

Expression profiles of these *AtGolS* genes, using the same plant materials as used for the sugar analysis, are shown in Figure 2(d). *AtGolS1* and *2* were induced by drought and high-salinity stresses but not by cold stress. By contrast, *AtGolS3* was induced by cold stress but not by drought or high-salinity stress. Although *AtGolS1* and *2* showed similar patterns of gene expression, the level of *AtGolS2* mRNA was higher than that of *AtGolS1* mRNA. In mature seeds, a high level of expression of *AtGolS1* and *2* was observed, whereas the expression of *AtGolS3* was hardly observed.

Enzymatic properties of the bacterially expressed AtGolS proteins

To examine whether the *AtGolS1*, *2* and *3* genes encode a galactinol synthase, we analysed the biochemical properties of the recombinant AtGolS1, 2 and 3 proteins expressed in *Escherichia coli*. The DNA fragments for the

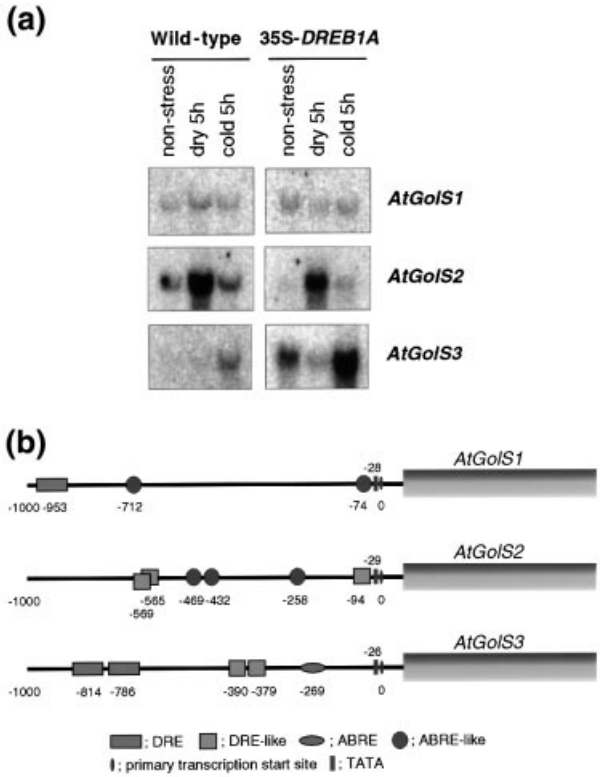


Figure 5. Expression of *AtGolS* genes in the transgenic *Arabidopsis* plants that overexpress *DREB1A* cDNA.
(a) Total RNA was prepared from 3-week-old *Arabidopsis* wild-type plants and 35S-*DREB1A* plants grown on GM plates that had been treated with drought (dry) or low temperature at 4°C (cold) or untreated (non- stress) at the indicated times. Each lane was loaded with 10 μg of total RNA. The RNA was fractionated on a 1% agarose gel, blotted onto a nylon membrane, and hybridized with [^{32}P]-labelled *AtGolS1*, *2 and 3* cDNAs as probes.
(b) Comparison of the promoter region of the *AtGolS* genes. DRE (TACCGACAT); DRE-like (CCGAC); ABRE (CACGTGGC); and ABRE-like (CACGTG, G-box sequence) motifs are shown in the 1000 bp upstream regions of 5′ termini of the full-length cDNA clones. Numbers with minus signs indicate nucleotides upstream of the 5′ terminus of the putative transcription start sites of *AtGolS* genes.

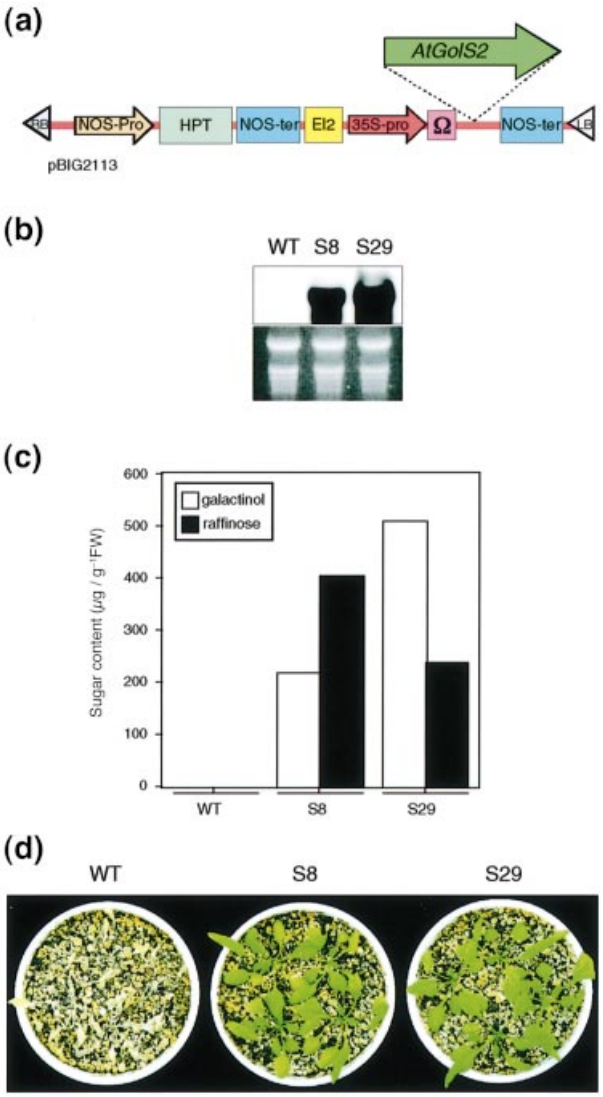


Figure 6. Creation and evaluation of the transgenic plants that overexpress sense *AtGolS2* cDNA.
(a) Constructs of the CaMV 35S-promoter::*AtGolS2* cDNA fusion genes.
(b) Expression of transgenes in the transgenic plants.
(c) Galactinol and raffinose content of the *AtGolS2* transgenic plants. Three-week-old plants were exposed to withholding water stress (dry), or to normal growth conditions (water) for 12 days. Control transgenic plants with the vector pBIG2113N (wild type); transgenic plants with 35S-*AtGolS2*-sense–29 (S29).
(d) Phenotype of plants exposed to drought stress. Three-week-old plants were exposed to drought stress, performed by withholding water for 14 days. After the drought stress plants were rehydrated for 5 days. Plants used for the analysis were transgenics with the vector pBIG2113N (wild type) as a control, 35S-*AtGolS2*-sense–8 (S8) and 35S-*AtGolS2*-sense–29 (S29)

AtGolS1, *2* and *3* coding regions were amplified by PCR and fused to the *GST* (glutathione *S*-transferase) gene in-frame using the pGEX4T-1 (Amersham Pharmacia Biotech, Uppsala, Sweden) to construct a chimeric plasmid pGST-AtGolS1, 2 and 3, respectively. The GST-AtGolS1, 2 and 3 proteins were overexpressed in *E. coli* and purified from the crude cell extract using a glutathione-Sepharose 4B.

We then examined whether the purified GST-AtGolS1, 2 and 3 recombinant proteins synthesize galactinol in the presence of myoinositol, a substrate of GolS. As shown in Figure 3, galactinol as well as myoinositol was detected after the incubation with GST-AtGolS1, 2 and 3 (Figure 3b–d), whereas only myoinositol was detected in the control (Figure 3a). These results show that *AtGolS1*, *2* and *3* encode galactinol synthase.

Expression analysis of the AtGolS *genes in* Arabidopsis *plants under stress conditions*

We analysed the expression of three *AtGolS* genes under various abiotic stress conditions using *Arabidopsis* plants grown on agar plates (Figure 4). *AtGolS1* and *2* were

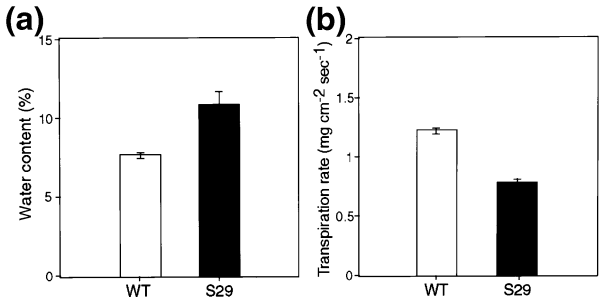


Figure 7. Water content of soil for the growth of transgenic plants, and transpiration rates of the *AtGolS* overexpressing transgenic plants.
(a) Water content of the soil for the growth of plants after drought stress. Three-week-old plants were exposed to drought stress, performed by withholding water for 14 days. Plants used for the analysis were transgenic with the vector pBIG2113N (wild-type) and the 35S-sense-*AtGolS2*-29 (*AtGolS2*-S-29) fusion genes.
(b) Transpiration rates of 5-week-old *AtGolS2* transgenic plants under normal growth conditions. Transpiration rates were measured using fully expanded leaves.

induced by drought and high-salinity stresses, but not by cold stress, whereas *AtGolS3* was strongly induced by cold stress, but not by drought or high-salinity stress. Although rice *wsi76*, a *GolS* homologue, is not induced by ABA (Takahashi *et al.*, 1994), *AtGolS1* and *2* were weakly induced by ABA but *AtGolS3* was not.

AtGolS3 is controlled by DREB1A

Overexpression of transcription factors *DREB1A/CBF3* (DREB: dehydration responsive-element binding factor; CBF: C-repeat binding factor) and *CBF1* improves the tolerance to drought, high salinity and cold stresses (Jaglo-Ottosen *et al.*, 1998; Kasuga *et al.*, 1999; Liu, Q. *et al.*, 1998), and causes accumulation of galactinol and raffinose in the transgenic plants (Gilmour *et al.*, 2000; our unpublished data). The *DREB1/CBF* genes are rapidly induced in response to low temperature, and encode transcriptional activators that control the expression of stress-inducible genes containing DRE in their promoters. Overexpression of *DREB1A/CBF3* in transgenic *Arabidopsis* plants has been shown to induce overexpression of many stress-inducible target genes under unstressed conditions, such as *RD* (responsive to dehydration), *ERD* (early responsive to dehydration), *COR* (cold-regulated), and *KIN* genes, and to enhance stress tolerance of the transgenic plants to drought, freezing and high-salinity stresses (Gilmour *et al.*, 2000; Kasuga *et al.*, 1999; Seki *et al.*, 2001). Then the expression of *AtGolS* genes in *DREB1A*-overexpressing transgenic plants (35S::*DREB1A* plants) was examined. As shown in Figure 5(a), the *AtGolS3* mRNA was significantly observed in the 35S::*DREB1A* plants under unstressed conditions, and accumulated greatly under cold-stress conditions. By contrast, *AtGolS1* and *2* mRNAs were unchanged in the 35S::*DREB1A* plants and did not accumulate under stress conditions. These results suggest that *AtGolS3* is a target gene of DREB1A, but the others are not. In the *AtGolS3* promoter, we found two DRE (TACCGACAT) and two DRE-like A/GCCGAC core motifs (Yamaguchi-Shinozaki and Shinozaki, 1994) at –814 and –786 bp, and at –390 and –379 bp, respectively, upstream from the transcription start site (Figure 5b). These results suggest that the increase in raffinose levels of *DREB1A*-overexpressing plants (Gilmour *et al.*, 2000) is due to the increased expression of *AtGolS3*, which may improve freezing tolerance of the transgenic plants. On the other hand, *AtGolS1* and *2* are not controlled by DREB1A.

Creation of AtGolS*-overexpressing transgenic plants and evaluation of their drought-stress tolerance*

To further analyse the role of *AtGolS* in the accumulation of galactinol and raffinose under drought-stress conditions, we generated transgenic *Arabidopsis* plants that overexpressed sense *AtGolS2* transcripts (Figure 6a). We selected two transgenic lines from overexpressors of *AtGolS2* (*AtGolS2*-S8, S29) which showed constitutive strong expression of each *AtGolS* transgene (Figure 6b).

Then we measured galactinol and raffinose contents in these transgenic plants under unstressed conditions. Under normal growth conditions, galactinol and raffinose were not detected in wild-type plants by HPLC analysis. In contrast, each sense transgenic plant showed significant accumulation of galactinol and raffinose under normal growth conditions (Figure 6c).

To examine whether altered expression of *AtGolS* genes affected the tolerance to drought stress in the transgenic plants, the *AtGolS2* sense transgenic plants (S8, S29) were grown for 3 weeks under normal conditions, and then exposed to drought stress by stopping water supply. At 14 days after drought-stress treatment, each *AtGolS2* sense transgenic plant clearly showed stronger stress tolerance to drought than the wild-type plants. When plants were rehydrated, all the sense transgenic plants recovered, but none of the control plants survived (Figure 6d). These results indicate important roles of galactinol and raffinose in drought-stress tolerance, and a key role of *AtGolS* in the production of galactinol and raffinose.

Water content of the soil and transpiration rate of the transgenic plants

We also measured the water content of the soil in pot-grown plants during dehydration. As shown in Figure 7(a), the water content of the soil in the pots used for growth of these *AtGolS* sense transgenic plants was higher than that of wild-type plants. This suggests that the drought toler-

ance of *AtGolS* sense transgenic plants is due to less transpiration of their leaves.

Then we measured the transpiration from leaves of the *AtGolS* sense transgenic plants and wild-type plants that had been grown under normal conditions (Figure 7b). It became clear that the transpiration rate of the *AtGolS* sense transgenic plants was lower than that of wild-type plants.

Discussion

Accumulation of galactinol and raffinose and expression of galactinol synthase genes during abiotic stress treatment

Accumulation of galactinol and RFO (raffinose and stachyose) was observed during seed maturation in *Arabidopsis* (Figure 1b), as in soyabean and maize (Blackman *et al.*, 1992; Brenac *et al.*, 1997). This suggests important roles of these sugars in the desiccation tolerance of mature seeds. We analysed these sugars in *Arabidopsis* rosette plants treated with drought, high-salinity and cold stresses, and showed that galactinol and raffinose, but not stachyose, accumulate in all the stress-treated plants. This suggests that galactinol and raffinose are involved in the stress tolerance in plants exposed to abiotic stress, as well as in mature seeds.

To examine the function of galactinol and raffinose in stress tolerance, seven genes for galactinol synthase (*GolS*), a key enzyme in RFO synthesis, were isolated based on the *Arabidopsis* genome sequence. Among these seven genes, three *GolS* genes were induced by drought, high-salinity or cold stress, and were named *AtGolS1*, *2* and *3* (Figure 2a). RNA gel-blot analysis revealed that *AtGolS1* and *2* are induced by drought and high-salinity stresses, but not by cold stress; but *AtGolS3* is induced by cold stress, but not by drought or high-salinity stresses (Figure 2d). This suggests that *AtGolS1* and *2* mainly function in drought and high-salinity stress tolerance, whereas *AtGolS3* functions in cold stress tolerance. These expression patterns resemble the relation between *DREB1* and *DREB2* which encode key transcription factors involved in cold and dehydration-responsive gene expression, respectively (Liu, Q. *et al.*, 1998). The expression pattern of *DREB1* is similar to that of *AtGolS3*, which is induced only by cold stress. In contrast, the expression pattern of *DREB2* is similar to that of *AtGolS1* and *2* that is induced by drought and high-salinity stresses, but not by cold stress (Liu, Q. *et al.*, 1998). In fact, we showed that *AtGolS3* is controlled by DREB1A (Figure 5). However, *AtGolS1* and *2* are not controlled by DREB1A. In this study we identified *AtGolS3* as a new target gene of DREB1A. Interestingly, *AtGolS3* was induced by cold stress but not by drought stress (Figures 4 and 5). Most of the DREB target genes are induced by both drought and cold stresses. *AtGolS3* is the first example of a DREB target gene that is induced only by cold stress. The *AtGolS3* promoter contains four DRE related motifs and one ABA-responsive element (ABRE). Combinations of these *cis*-acting elements may be involved in cold-specific expression of *AtGolS3*. Another possibility is that some negative factor induced during dehydration stress may repress the drought-responsive expression of *AtGolS3*.

AtGolS1 and *2* were slightly induced by exogenous ABA treatment. The *AtGolS1* and *2* promoters have two ABREs which may function in ABA-responsive expression, whereas the *AtGolS3* promoter has one ABRE (Figure 5b). *AtGolS3* is not induced by ABA, which indicates that one ABRE is not functional in the promoter.

Drought tolerance of AtGolS-*overexpressing transgenic plants and a key role of* AtGolS *in the production of galactinol and raffinose*

To examine the function of galactinol and raffinose as osmoprotectants in plants under water-deficit stresses, we created *AtGolS2*-overexpressing transgenic *Arabidopsis* plants. In the transgenic plants, not only was the expression of *AtGolS* genes increased, but also galactinol and raffinose were accumulated (Figures 6b,c). The leaves of the transgenic plants under normal growth condition contained both galactinol and raffinose at a concentration of 200–500 $\mu g\ g^{-1}$ FW. This content was equivalent to that in the transgenic plants in which proline or glycinebetaine was superfluously accumulated (Hayashi *et al.*, 1997; Nanjo *et al.*, 1999b). These transgenic plants also showed drought-stress tolerance (Figure 6d). Thus we showed that galactinol synthase has a key role in controlling galactinol and raffinose in plants, and that galactinol and raffinose function in drought tolerance. Transgenic plants that overexpressed *DREB1A/CBF3* are tolerant to dehydration and cold stress (Kasuga *et al.*, 1999; Liu, Q. *et al.*, 1998), and accumulate more galactinol and raffinose as well as other sugars in comparison with the wild-type plant (Gilmour *et al.*, 2000; our unpublished data). The transgenic plant accumulates proline as well as galactinol and raffinose (Gilmour *et al.*, 2000; our unpublished data). These results suggest that galactinol and raffinose function as osmoprotectants like proline.

We measured the water content in the soil in pot-grown plants under drought stress, and showed that the water content of *AtGolS* sense transgenic plants is higher than that of wild-type plants. The transpiration rate of the *AtGolS* sense transgenic leaves was significantly suppressed in comparison with that of vector control plants. It is well known that ABA suppresses transpiration of leaves by closing stomata; the synthesis of ABA may be activated by galactinol or raffinose, which causes stomata closure.

Transgenic plants overexpressing *AtNCED3* cDNA accumulate ABA, which causes stomatal closure making plants drought tolerant (Iuchi *et al.*, 2001). The *AtNCED3* gene is an *Arabidopsis* drought-inducible gene encoding 9-*cis* epoxycarotenoid dioxygenase (NCED), a key enzyme in ABA biosynthesis. The phenotype of the *AtNCED3*-overexpressing plants was similar to that of the *AtGolS*-overexpressing plants (data not shown).

Using isolated chloroplast membranes of spinach (*Spinacia oleracea*), raffinose was suggested to reduce the inactivation of electron (DCIP reduction) and cyclic photophosphorylation in photosynthesis under freezing, drought and high-temperature stresses (Santarius, 1973). Furthermore, Santarius and Milde (1977) reported accumulation of sucrose and raffinose in chloroplasts of frost-hardy leaves. Thylakoid membranes may be partially protected by raffinose in chloroplasts. This membrane stabilization depends on the concentration of sugars and their molecular size. The trisaccharide raffinose may be more effective in membrane stabilization than either disaccharide sucrose or monosaccharide glucose.

Experimental procedures

Plant material and growth conditions

Arabidopsis thaliana ecotype Columbia was used in this study. Wild-type seeds were sown in 9 cm plastic pots filled with a 1 : 1 mixture of perlite/vermiculite and watered with 1000-fold diluted Hyponex (Hyponex, Osaka, Japan). Transgenic plants were grown on germination medium (GM) agar plates (Valvekens *et al.*, 1988) containing 30 mg l^{-1} hygromycin (GMH) or 30 mg l^{-1} kanamycin (GMK). Two-week-old plate-grown plants were transferred to soil pots. The plants were then grown under continuous illumination of approximately 2500 lux at 22°C.

Stress treatment for agar plate-grown plants

Three-week-old plate-grown plants were harvested from GM agar plates, then dehydrated on Whatman 3MM paper at room temperature and approximately 60% humidity under dim light. Plants subjected to treatment with ABA and salt stress were grown hydroponically in solution containing 100 mM ABA and 250 mM NaCl, respectively, under dim light. Cold treatment was conducted under dim light by exposure of plants grown at 22°C to a temperature of 4°C. In each case, plants were subjected to the stress treatments for various periods and were frozen in liquid nitrogen.

Stress treatment for soil-grown plants

Dehydration, salinity and cold stresses were imposed by transferring 3-week-old soil-grown plants to vats without sufficient water, to 1000-fold diluted Hyponex containing 150 mM NaCl, and to 4°C with 1000-fold diluted Hyponex under continuous illumination, respectively, for 0, 3, 7 and 10 days.

Isolation of galactinol synthase genes from Arabidopsis

Seven open reading frames that have sequence similarity with *GolS* genes were identified from the *Arabidopsis thaliana* ecotype Columbia databases (AtDB, Stanford University; http://genome-http://www.stanford.edu/Arabidopsis/) by a homology search against *Oryza sativa GolS* gene homologue *wsi76* (Takahashi *et al.*, 1994) using the BLAST program. Genomic DNA was isolated from *Arabidopsis* plants. Synthetic oligonucleotides for PCR based on the sequences in the DNA database were used to isolate *GolS* genes. The synthetic oligonucleotides were as follows: 5′-CAAGGATCCGCAGATCACGTGCTAATCAC-3′ and 5′-CAAGGATCCCCTGGCAATCAAGCAGCGGA-3′ (set A; accession number AC002337); 5′-CGCCACAGTACAAGATCGGTTA-3′ and 5′-CATGAAGAGGCGTATGCAGC-3′ (set B; accession number AC009323); 5′-CTTTCTCGGACAAGATGGCA-3′ and 5′-GTGTTGACAAGAACCTCGCT-3′ (set C; accession number AC003970); 5′-GGCCCCTGAGATTTCCGTAA-3′ and 5′-GCAGGAAAGTAGGTCATTTCTG-3′ (set D; accession number AC002292); 5′-GATTGTCGAGAAGAGGATCG-3′ and 5′-GGAAGGGGCAAGGCTTGTTA-3′ (set E; accession number AB005244); 5′-GGCTCAAATGTCGATGACCGTCGAGAAGAG-3′ and 5′-GCAGCGGAAGGGGCAAGACTAATAAGGAC-3′ (set F; accession numbers AL049171and AL161564); and 5′-CAAGGATCCAAGGTGCAGCAGGAGAGTAGG-3′ and 5′-CAAGGATCCTGCATCCGAGAAGGCTCCTAA-3′ (set G; accession number AC004473). DNA fragments of putative *GolS* genes were amplified from the *Arabidopsis* full-length cDNA library (Seki *et al.*, 1998; Seki *et al.*, 2001) by PCR using the synthetic oligonucleotide sets A–G, respectively. Their DNA fragments were cloned into the pBluescript II SK^+ cloning vector (Stratagene, La Jolla, CA, USA). Three cDNAs for stress-inducible *GolS* genes were isolated by screening the *Arabidopsis* full-length cDNA library using the cDNA fragments that were PCR-amplified with the synthetic oligonucleotide sets A–C as probes, and named *AtGolS1*, *2* and *3*, respectively.

Northern analysis

Total RNA was isolated according to the method described by Nagy *et al.* (1988). Total RNA was fractionated in a 1% agarose gel containing formaldehyde and was blotted onto a nylon filter (Sambrook *et al.*, 1989). The filters were hybridized in DIG Easy Hyb (Roche Diagnostics, Indianapolis, IN, USA) at 68°C with DIG-labelled fragments using DIG RNA Labeling Kit (SP6/T7) (Roche Diagnostics); the probes were PCR-amplified from the first-strand cDNAs by gene-specific primers. Following hybridization, the filters were washed twice in 2 × SSC, 0.1% SDS for 5 min at room temperature and twice in 0.5 × SSC, 0.1% SDS at 68°C for 15 min. The washed filters were developed with an immunostaining system using a DIG DNA detection kit (Roche Diagnostics).

In Northern blot analysis, filters were hybridized with [^{32}P]-labelled fragments at 42°C, washed twice with 0.1 × SSC, 0.1% SDS at 65°C for 15 min, and autoradiographed.

Expression of GST-AtGolS *gene fusion proteins*

Full-length cDNA fragments of *AtGolS1*, *2* and *3* were amplified by PCR using synthetic oligo-DNAs 5′-CGCGGATCCATGGCTCCGGGGCTTACTCAAAC-3′ and 5′-CGCGGATCCCCACCGACAATTTTAACTCCTGG-3′ (*AtGolS1*); 5′-CGCGGATCCATGGCACCTGAGATCAATACC-3′ and 5′-CGCGGATCCGAGGCGTATGCAGCAACGAGC-3′ (*AtGolS2*); and 5′-CGCGGATCCATGGCACCTGAGATGAACAACAAGTTG-3′ and 5′-CGCGGATCCCTGGTGTTGACAAG-

AACCTCGCTC-3′ (*AtGolS3*). The PCR fragments were cloned into the *Eco*RV site of pBluescript II SK$^+$. The fragments were isolated from the resultant plasmids by *Eco*RI digestion, and inserted into the *Eco*RI site of pGEX4T (Amersham Pharmacia Biotech AB, Uppsala, Sweden) to yield *pGST–AtGolS1, 2 and 3*. These chimeric genes were expressed in *E. coli*. We have previously described the procedures for the purification of the fusion protein in detail (Iuchi *et al.*, 2000).

Assay of galactinol synthase activity

The enzyme activity of galactinol synthase was assayed as described (Liu *et al.*, 1995). The reaction mixture (1 ml) contained buffer (50 mM Hepes–Na, 2 mM DTT pH 7.0), 4 mM $MnCl_2$, 4 mM UDP-Gal, 20 mM myoinositol, 160 μg BSA and 0.017 mg enzyme purified as above. The enzyme was pre-incubated with the reaction mixture at 30°C for 15 min and then incubated with the substrates at 30°C for 30 min. The reaction was stopped by adding 2 ml ice-cold 100% ethanol and centrifuging in a micro-centrifuge. The extract was dried up, and products were analysed by high-performance liquid chromatography (HPLC) using the sugar analysis system DX500 (CarboPac MA1, Sunnyvale, CA, USA) and a pulsed amperometry detector (Dionex Corporation, Sunnyvale, CA, USA).

Sugar analysis of plants

The plant cell extract was prepared from various plant tissues. Plant tissues were frozen with liquid nitrogen, crushed, added to 10 ml 80% ethanol preheated to 80°C, then boiled for 10 min at 90°C. Then the series of steps was repeated twice (a total of three rounds of these steps was performed). Next, sugars of the cell extracts were analysed by HPLC as described above.

Construction of transgenic plants

To generate transgenic plants with sense *AtGolS* cDNA, we constructed chimeric genes in which the coding sequences of the *AtGolS2* cDNA were fused in a sense orientation between the cauliflower mosaic virus 35S promoter and the *nos* terminator sequence of the pBIG2113Not vector in which the kanamycin-resistance gene (NPT-II) was replaced with the hygromycin-resistance gene (HPT) of the pBE2113Not expression vector (Liu, Q. *et al.*, 1998; Mitsuhara *et al.*, 1996), and named the plasmids 35S-AtGolS2 (sense). We introduced these plasmids into wild-type *Arabidopsis* seedlings by *Agrobacterium*-mediated transformation. T_2 seeds were used for subsequent experiments.

Measurement of leaf transpiration rate

The transpiration rate was measured in fully expanded leaves with a portable photosynthesis system (model LI-6400, Li-Cor, Lincoln, NE, USA) under the following conditions: 100 μmol m^{-2} sec^{-1}, 350 p.p.m. CO_2, 22°C and 70% relative humidity.

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