Results
Part I: Accumulation of galactinol and RFO during water deficit stress in *Arabidopsis* plant and several tissues

*Galactinol and RFO contents in several tissues*

I measured the accumulation of endogenous levels of galactinol and RFO in *Arabidopsis* reproductive organs or soil-grown *Arabidopsis* plants during dehydration, high-salinity and cold stresses. As shown in Figure 2, 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 soybean (*Glycine max*) (Blackman et al., 1992).

*Accumulation of galactinol and RFO during water deficit stress in Arabidopsis plant*

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. While the unstressed plants had no detectable amount of RFO, galactinol and raffinose accumulated in all the stress-treated plants (Figure 3). O-methyl-inositol that functions in *Coleus* as osmoprotectant was not detected under these stresses in *Arabidopsis* (Pattanagul and Madore, 1999). These results imply that galactinol and raffinose function in plants under osmotic stress as a osmoprotectant. However, stachyose, the first major RFO in seeds, did not accumulate in these stressed plants. For myo-
inositol which is a substrate of galactinol, the significant accumulation under dehydration and high-salinity stress was not recognized, though that was observed only under cold stress (Figure 4). The content of sucrose which was a substrate of raffinose increased by dehydration, high-salinity and cold stresses (Figure 4). For glucose and fructose, though, the rapid increasing of accumulation was observed by only cold stress, it was not recognized under drought and high salinity stresses (Figure 4). Thus, it was anticipated that glucose and fructose did not function for the acquisition of the water deficit stress resistance.
Part II: Expression and functional analysis of galactinol and raffinose synthase genes from Arabidopsis

Isolation and the sequence analysis of GolS genes from Arabidopsis

Seven GolS-related genes were identified from the A. thaliana (Columbia) genome databases (AtDB, Stanford University; http://www.stanford.edu/Arabidopsis/) by a homology search against the Oryza sativa GolS gene homolog, wsi76 (Takahashi et al., 1994) using the BLAST program. Their GenBank accession numbers are AC002337, AC009323, AC003970, AC002292, AB005244, AL049171/AL161564, and AC004473. I 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 “Materials and Methods” for details), and cloned into pBluescript vector. To investigate the stress-inducibility of the 7 AtGolS genes, I analyzed the expression of these AtGolS genes under various abiotic stress conditions using Arabidopsis plants grown on agar plates (Figure 5). Among 7 AtGolS genes, three genes were induced by drought or cold 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. Phylogenetic analysis revealed that AtGolS1, 2 and 3 are highly homologous with each other (Figure 6). All of them are also highly homologous to known GolS genes from Ajuga reptans (Sprenger and Keller, 2000), soybean (Kerr et al.,
1993), zucchini (Kerr et al., 1993) and rice (Takahashi et al., 1994) (Figure 7). 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.

**Verification of the specificity of the probe for Northern analysis**

Cross hybridization by the expression analysis was considered, because the homology between AtGolS genes was very high. So full-length cDNA probe and the partial specific cDNA probe with 3' flanking region were made, to verify hybridization specificity. The hybridization was carried out by the DIG labeled probe using membrane on which synthesized RNA by RNA polymerase from several AtGolS genes was blotted. It revealed that AtGolS1, 2, 3 RNAs were specifically recognized by AtGolS1, 2, 3 probes, respectively, when the full length probe or 3' end region probe were used (Figure 8). Therefore, the Northern analysis was carried out using the full-length probe in the following experiments because the signal of full length probe was stronger than that of 3' end region probe in AtGolS2.

**Expression profiles of these AtGolS genes in soil-grown plants and in several tissues**

To investigate the stress-inducibility and the tissue-specific expression of the 7 AtGolS genes I carried out the Northern blot analysis using the same plant materials as used for the sugar analysis (Figure 9). 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. Though AtGolS1 and 2 showed similar patterns of gene expression, the level of AtGolS2 mRNA was higher than that of AtGolS1 mRNA. In mature seeds in which RFO contents were very high, a high level of expression of AtGolS1 and 2 was observed, whereas the expression of AtGolS3 was hardly observed. Thus, it revealed that the time course of the expression of AtGolS genes correlated with the time course of the accumulation of galactinol and RFO tightly.

*Enzymatic properties of the bacterially expressed AtGolS proteins*

To examine whether the AtGolS1, 2 and 3 genes encode a galactinol synthase, I analyzed the biochemical properties of the recombinant AtGolS1, 2 and 3 proteins expressed in *E. coli*. The DNA fragments for the AtGolS1, 2 and 3 coding regions were amplified by PCR and fused to the GST gene in frame using the pGEX4T-1 (Pharmacia) to construct a chimeric plasmid pGST-AtGolS1, 2 and 3, respectively (Figure 10). The GST-AtGolS1, 2 and 3 proteins were overexpressed in *E. coli*, and purified from the crude cell extract using a glutathione-Sepharose 4B (Figure 11).

I then examined whether the purified GST-AtGolS1, 2 and 3 recombinant proteins synthesize galactinol in the presence of myo-inositol, a substrate of GolS. As shown in Figure 12, galactinol as well as myo-inositol was detected after the incubation with GST-AtGolS1, 2 and 3 (Figure 12b, c and d), whereas only myo-inositol was detected in the control (Figure 12a). These results show that AtGolS1, 2 and 3 encode galactinol synthase. The enzymatic activity of AtGolS was equivalent to galactinol synthase activity of kidney been (Liu *et al.*, 1995).
Expression analysis of the AtGolS genes in Arabidopsis plants under stress conditions

I analyzed the expression of three AtGolS genes under various abiotic stress conditions using Arabidopsis plants grown on agar plates (Figure 13). AtGolS1 and 2 were 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 wsl76, a GolS homolog, is not induced by ABA (Takahashi et al., 1994), AtGolS1 and 2 were weakly induced by ABA, but AtGolS3 was not.

Isolation and characterization of raffinose synthase gene in Arabidopsis

I identified a raffinose synthase gene which in Arabidopsis genome functions in the next step of galactinol synthase for RFO synthesis, I isolated a full-length cDNA for raffinose synthase, and named AtRafS1 (Arabidopsis thaliana raffinose synthase). Figure 14 showed the alignment of the amino acid sequence between AtRafS1 and CsRafS1, the raffinose synthase gene isolated from Cucumis sativus (accession No. AC007138). On the Arabidopsis genome, there is only one raffinose synthase gene that has high sequence similarity with known raffinose synthase gene.

In order to examine whether AtRafS1 is induced by stress treatment, I analyzed the expression of AtRafS gene under various abiotic stress conditions using Arabidopsis plants grown on agar plates. The AtRafS1 gene was induced not only by drought and high salinity stresses but also by cold stress and ABA treatment (Figure 15). In contrast, the AtGolS genes are classified into 2 groups, that is, a drought- and high salinity-inducible gene group and a cold-specific inducible gene group.
Part III: Regulation of the expression of AtGols genes under water-deficit stress

Regulation of the expression of AtGols genes

The expression patterns of genes induced by drought have been analyzed by Northern analysis. There are broad variations in the timing of induction of these genes under drought conditions. Most of the drought-inducible genes are induced by high salinity stress. Most of the drought-inducible genes are also induced by respond to cold stress but some of them are not. Many genes respond to ABA whereas some others do not. There are at least four independent signal transduction pathways between the perception of water deficit stress signal and gene expression. Two of them are ABA-dependent (Pathways I and II) and two are ABA-independent (Pathways III and IV) (Figure 16). One of the ABA-dependent pathways requires protein biosynthesis (Pathway I). Cis- and trans-acting factors involved in ABA-induced gene expression have been extensively analyzed in one of the ABA-dependent pathway that does not require de novo protein biosynthesis (Pathway II). One of the ABA-independent pathways overlaps with that of the cold response (Pathway IV). There are several drought-inducible genes that do not respond to either cold or ABA treatment, which suggests that there is a fourth pathway in the dehydration stress response (Pathway III).

AtGols1, 2 were induced by ABA treatment, whereas AtGols3 was induced by cold stress but not ABA based on the Northern analysis (Figure 13). This result suggests that AtGols1 and 2 is controlled by ABA-dependent pathway (Pathways I or II) and AtGols3 is controlled by ABA independent pathway (Pathway IV).
Sugar analysis of the DREB1A overexpression plant

DRE binding protein, DREB1/CBF and DREB2 which bind dehydration responsive element, DRE function as transcription factors in ABA independent pathway (Pathway IV). Expression of the DREB1A/CBF3 gene was induced by only cold stress but not by drought and high-salinity stresses, whereas expression of the DREB2A gene was induced by drought and high-salinity stresses but not by cold stress. Expression of DREB1A/CBF3 shows similar pattern with that of AtGolS3, while DREB2A shows similar expression pattern with those of AtGolS1 and 2. Overexpression of transcription factors, DREB1A/CBF3 and DREB1B/CBF1, improves the tolerance to drought, high salinity and cold stresses (Liu Q. et al., 1998, Jaglo-Otto sen et al., 1998, Kasuga et al., 1999). 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, ERD, COR (cold-regulated), and KIN genes, and to enhance stress tolerance of the transgenic plants to drought, freezing and high-salinity stresses (Kasuga et al., 1999, Gilmour et al., 2000, Seki et al., 2001). However, it has not been solved how DREB1A/CBF3 overexpressing plants significantly acquire stress tolerance to drought and freezing.

Recently, it was revealed that DREB1A/CBF3 overexpression plants accumulated more proline and sugars, especially raffinose as compared to wild type plants by Thomashow and me (Figure 17). Accumulated proline and sugars may be involved in improving water
stress resistance in the *DREB1A* overexpression plant.

*AtGolS3* is controlled by *DREB1A*

It was suggested that *AtGolS* genes, which catalyzes the rate-determining step of the raffinose synthesis, were controlled by *DREB1A*, because raffinose superfluously accumulated in the *DREB1A* overexpression plant. Then, I examined the expression of *AtGolS* genes in *DREB1A*-overexpressing transgenic plants (*35S::DREB1A* plants). As shown in Figure 18, the *AtGolS3* mRNA was significantly observed in the *35S::DREB1A* plants under unstressed conditions and greatly accumulated under cold-stress conditions. By contrast, *AtGolS1* and 2 mRNAs were unchanged in the *35S::DREB1A* plants and not accumulated under stress conditions. These results suggest that *AtGolS3* is a target gene of *DREB1A* but the others are not. In the *AtGolS3* promoter, I found 2 DRE (TACCGACAT) and 2 DRE-like A/GCGAC core motifs (Yamaguchi-Shinozaki and Shinozaki, 1994) at −814 bp and −786 bp, and at −390 bp and −379 bp upstream from the transcription start site, respectively (Figure 19). 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*. The *AtGolS2* promoter has 3 DRE-like and 3 ABRE-like cis-acting elements whereas the *AtGolS1* promoter contains only ABRE (Figure 19).

This result indicated that *AtGolS1* and 2 existed in ABA-dependent pathway (Pathway II) and suggested that *AtGolS2* also existed in ABA-independent pathway through *DREB2A*.
(Pathway IV). Furthermore, it was indicated that AtGolS3 is controlled by ABA independent pathway through DREB1A from the Northern analysis in the 35S::DREB1A plants.
Part IV: Functional analysis of AtGols genes in plant level

Creation of the transgenic plants with sense AtGols cDNAs

Generally, the functional analysis of the subject gene in individual plant level is achieved by studying the phenotype of the transgenic plants in which the subject gene was overexpressed and suppressed. To begin with, I generated antisense transgenic Arabidopsis plants with AtGols1, 2 and 3, respectively. However, the transgenic line in which the accumulation of galactinol and raffinose sufficiently decreased was not selected, though their expression levels were decreased. To elucidate the reason, the expression analysis of AtGols genes in the AtGols antisense plants was carried out. The expressions of AtGols2 and AtGols3 were not suppressed, but the expression of AtGols1 was suppressed in the AtGols1 antisense plants (Figure 20). Similar phenomenon was observed even in the antisense plants of AtGols2 and AtGols3. Thus, the contents of galactinol and raffinose sufficiently did not decrease in the AtGols antisense plants, since the expression of other AtGols genes could not be suppressed.

Then, I analyzed the function of AtGols genes using AtGols overexpressing transgenic Arabidopsis plants. To generate the transgenic plants with sense AtGols cDNA, I constructed chimeric genes in which the coding sequences of the AtGols1, 2 or 3 cDNAs were fused in a sense orientation between the cauliflower mosaic virus 35S promoter and the nos terminator sequence of the pBIG2113Not vector in which kanamycin resistance gene (NPT-II) was replaced with the hygromycin resistance gene (HPT) of the pBE2113Not expression vector (Liu, Q. et al., 1998; Mitsuhabara et al., 1996), and named the
plasmids 35S-AtGolS1, 2 or 3 (sense) (Figure 21). I introduced these plasmids into wild-type Arabidopsis seedlings by Agrobacterium-mediated transformation. T2 seeds were used for subsequent experiments.

*Analyses of expression level and galactinol and raffinose quantity of the transgenic plant*

Expression of the AtGolS cDNA transgenes were analyzed in generated transgenic plants in order to select the transgenic lines which showed high level expression of the transgenes. Transgenic plants with the empty vector was used as a control (wild type). As the result of Northern analysis, I selected 2 transgenic lines from overexpressors of AtGolS2 (AtGolS2-S8 and S29) which showed constitutive strong expression of each AtGolS transgene (Figure 22).

Then, I 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 23). The galactinol and raffinose contents of both the S8 and S29 tranagenics were equivalent to those of wild type exposed to drought, high salinity or cold stress for 14 days as shown in Figure 3.

*Evaluation of drought-stress tolerance of the AtGolS2 overexpressing transgenic plants*
To examine whether altered expression of AtGols gene affected the tolerance to drought stress in the transgenic plants, the AtGols2 sense transgenic plants (S8 and 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 (S8 and S29) 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 24). 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**

I measured the water content of the soil in pot-grown plants during dehydration. As shown in Figure 25, 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 tolerance of AtGols sense transgenic plants is due to less transpiration of their leaves.

Then, I measured the transpiration from leaves of the AtGols sense transgenic plants and wild-type plants that had been grown under normal conditions (Figure 26). The transpiration rate of the AtGols sense transgenic plants was two-thirds of wild-type plants.

**Full-length cDNA microarray analysis**

Recently, it became clear that proline functions not only as an osmolyte but also as a
regulatory molecule of gene overexpression (Kiyosue et al., 1996, Nakashima et al., 1998, Satoh et al., unpublished data). Thus, I used the full-length cDNA microarray to identify stress-inducible genes controlled by the AtGolS genes. I used mRNA samples prepared from transgenic Arabidopsis plants that overexpress the AtGolS2 cDNA under the control of the CaMV 35S promoter (35S:AtGolS2 transgenic plants) and wild-type control plants (transformed with the vector pBIG2113N) for the preparation of Cy3-labeled and Cy5-labeled cDNA probes, respectively. These cDNA probes were mixed and hybridized with the full-length cDNA microarray (Seki et al., 2001). As a result, I could detect no cDNA clones with expression ratio (35S:AtGolS2 transgenic plants/wild-type control plants) greater than three times or less than $1/3$, which was the general condition for widely identifying the purpose genes, and observed no significant change in the expression profiles between the AtGolS2 sense transgenic plants and wild-type control (data not shown). These results suggest that galactinol and raffinose do not function as regulatory molecules of gene expression.