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 haigh 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://genome-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. Phylogenic 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 reptants* (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' flanling 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 speficically 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 *wsi76*, 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-Ottosen *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 aquire stress tolerance to drought and freezing.

Recently, it was revealed that *DREB1A/CBF3* overexpression plants accumulated more proline and sugars, especially raffinose as compered 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/GCCGAC 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; Mitsuhara *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. T_2 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 analyzeded 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 trangenics 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-third of wild-type plants.

Full-length cDNA microarray analysis

Recently, it became clear that proline functions not only as a 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.