

Discussion

Part I: Accumulation of galactinol and raffinose, and the 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 2) as in soybean and maize (Brenac *et al.*, 1997, Blackman *et al.*, 1992). This suggests important roles of these sugars in the desiccation tolerance of mature seeds. I analyzed 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. The accumulation of galactinol and raffinose under these stresses was equivalent to proline quantity that accumulates under the same stress conditions (Nanjo *et al.*, 1999A). In Figure 4, the rapid accumulation of glucose, fructose and sucrose was observed only under cold stress but not by drought and high salinity stresses. By contrast, the accumulation of osmolytes including galactinol and raffinose was slow under cold stress.

To examine the function of galactinol and raffinose in stress tolerance, 7 genes for galactinol synthase (*GolS*), a key enzyme of RFO synthesis, were isolated based on the *Arabidopsis* genome sequence. Among these 7 genes, 3 *GolS* genes were induced by drought, high salinity or cold stress, and were named *AtGolS1*, 2 and 3 (Figure 5). RNA gel blot analysis revealed that *AtGolS1* and 2 are induced by drought and high salinity stresses but not by cold stress, whereas *AtGolS3* is induced by cold stress but not by drought or high salinity stress (Figure 9). 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 very well to the relation between *DREB1* and *DREB2* that 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* that 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, I showed that *AtGolS3* is controlled by DREB1A (Figure 18). 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 9 and 13). Most of the DREB target genes are induced by both drought and cold stresses (Kasuga *et al.*, 1999, Seki *et al.*, 2001). *AtGolS3* is the first example of a DREB target gene that is induced only by cold stress. The *AtGolS3* promoter contains 4 DRE related motifs and 1 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*. On the other hand, *AtGolS1* and 2 are not controlled by DREB1A. However, *AtGolS2* may be controlled by DREB2A, because *AtGolS2* promoter has DRE, though *AtGolS2* is not controlled by DREB1A (Figure 18).

AtGolS1 and 2 were slightly and transiently induced by exogenous ABA treatment. The *AtGolS1* and 2 promoters have two ABREs, which may function in ABA-responsive expression, whereas *AtGolS3* promoter has one ABRE (Figure 19). *AtGolS3* is not

induced by ABA, indicating that one ABRE is not functional in ABA-responsive expression. The expression induced by ABA treatment was reached to maximum at 2-5 hours, and then decreased. The similar expression pattern was observed in several ABA-inducible genes, such as *RD29A*. Thus, osmotic stress signal is probably necessary on early stage of drought- and high-salinity- stress response and ABA biosynthesis, and then ABA plays important roles in the late stage of stress response and gene expression.

The Northern analysis and promoter analysis indicated that *AtGolS1* and 2 are regulated by ABA-dependent pathway (Pathway II) and suggested that *AtGolS2* is also regulated by ABA-independent pathway through *DREB2A* (Pathway IV-1). Furthermore, *AtGolS3* was shown to be controlled by ABA-independent pathway through *DREB1A* based on the Northern analysis using the 35S::*DREB1A* plants (Pathway IV-2) (Figure 27).

I showed that the *AtRafS1* gene is induced not only by drought and high salinity stresses but also by cold stress and ABA treatment (Figure 15), whereas *AtGolS* genes are differentially controlled by drought, high-salinity and cold-stresses. On the *Arabidopsis* genome, there is only one raffinose synthase gene that has high sequence similarity with known raffinose synthase. Therefore, *AtRafS1* plays important roles not only in drought and high-salinity stress but also in cold stress.

The microarray analysis on rehydration treatment after drought stress revealed that the expression levels of *AtGolS* genes and *AtRafS1* decreased by rehydration after dehydration (Oono *et al.*, unpublished data). Raffinose is decomposed to galactose and sucrose by α -galactosidase, or is decomposed in melibiose and fructose by invertase. It was known that α -galactosidase activity increases during water absorption of soybean

seeds, and that the accumulated raffinose decreases during seed maturation (Guimaraes et al., 2001). Therefore, I think that the decomposition of raffinose is induced similarly during rehydration of the water stressed plants.

Part II: Drought tolerance in *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, I created *AtGolS2*-overexpressing transgenic *Arabidopsis* plants. In the transgenic plants, not only the expression of *AtGolS2* genes increased but also galactinol and raffinose accumulated (Figures 22 and 23). The leaves of the transgenic plants under normal growth condition contained both galactinol and raffinose at a concentration of 200~ 500 µg/g fresh weight. The content of galactinol and raffinose was equivalent to those of proline or glycinebetaine accumulated in the transgenic plants that overexpress antisense *ProDH* and sense choline oxidase gene, respectively (Nanjo *et al.*, 1999b, Hayashi *et al.*, 1997). These *AtGolS2* overexpressing transgenic plants also showed drought-stress tolerance (Figure 24). Thus, I showed that galactinol synthase has a key role in controlling galactinol and raffinose contents in plants, and that galactinol and raffinose function in drought tolerance. Transgenic plants that overexpressed *DREB1A/CBF3* are tolerant to dehydration and cold stress (Liu, Q. *et al.*, 1998, Kasuga *et al.*, 1999), and accumulate more galactinol and raffinose as well as other sugars in comparison with those of 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. The *AtGolS2* overexpressing transgenic plants showed normal phenotype during growth under normal condition, though *DREB1A/CBF3* overexpressing plants were remarkably dwarf.

I measured the water content in the soil in pot which transgenic plants were grown under drought stress, and showed that the water content of *AtGolS2* sense transgenic plants is higher than that of wild-type plants. Transpiration rate of the *AtGolS2* 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 stomata closure making them 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). Recently cDNA microarray analysis has become a useful method to identify stress-inducible genes and target genes of stress-related transcription factors (Seki *et al.*, 2001, Kawasaki *et al.*, 2001). Therefore, I carried out microarray analysis, since galactinol and raffinose may function as signaling molecules and control the ABA-inducible genes. However, I could not detect any genes that are significantly upregulated or downregulated in the *AtGolS2*-overexpressing plants, which suggests that galactinol and raffinose do not function as signaling molecules but function as osmoprotectants.

Using isolated chloroplast membranes of spinach (*Spinacia oleracea* L.), 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.

Now, it is not clear whether galactinol or raffinose is more effective for water-deficit tolerance of plants. To solve this problem, I am planning to create transgenic plants which superfluously accumulates only galactinol by introducing antisense *AtRafSI* cDNA into the *AtGolS2* overexpressing transgenic plant and transgenic plant which superfluously accumulates only raffinose by introducing sense *AtRafSI* into wild type plant, and then compare drought resistance, between those transgenic plants.

In this study, I showed that *AtGolS2* overexpressing plants improve the drought tolerance. I also produced *AtGolS* antisense transgenic plants to reduce RFO in the transgenics. However, the contents of galactinol and raffinose did not decrease sufficiently, which may be due to other *AtGolS* genes unsuppressed in the transgenics. On the other hand, I tried to isolate the *AtGolS* genes mutants by screening of T-DNA insertional mutants. In the *Arabidopsis*, many T-DNA tag lines has been produced, and it is possible to isolate the disruptant mutant by the PCR screening. In this study, I screened 36,000 available T-DNA insertional mutant lines. However, the *AtGolS* knock-out mutants could not be isolated. Recently, it was reported that gene silencing using dsRNA was very effective to suppress the gene expression (Smith *et al.*, 2000). I am planning to create the transgenic plants that suppress the expression of *AtGolS* and *AtRafS* genes by dsRNAi, and

to evaluate its drought tolerance.

This study provides a direct evidence that the stress-inducible *AtGolS* gene controls the level of RFO, and that galactinol and raffinose play important roles in drought-stress tolerance, and that galactinol and raffinose functions as osmolytes involved in water-deficit-stress tolerance like other osmolytes such as glycine, betaine, mannitol, ononitol, trehalose, fructan and proline.