Materials and Methods

Plant material and growth conditions

Arabidopsis thaliana (L) Heynh. 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 plate (Valvekens *et al.*, 1988) containing 30 mg l^{-1} of hygromycin (GMH) or 30 mg l^{-1} of kanamycin (GMK). Two-week-old plate-grown plants were transferred to the 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, and then dehydrated on Whatman 3MM paper at room temperature and approximately 60% humidity under dim light. Plants subjected to treatment with abscisic acid (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, the 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, 10 days.

Isolation of galactinol synthase genes from *Arabidopsis*

Seven open reading frames that have sequence similarity with *GolS* genes were identified from the *A. thaliana* ecotype Columbia databases (AtDB, Stanford University; HTTP://genome-HTTP://www.stanford.edu/Arabidopsis/) by a homology search against *Oryza sativa GolS* gene homolog, *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'-CAAGGATCCCCTGGCAA TCAAGCAGCGGA-3' (set A; accession number AC002337); 5'-CGCCACAGTACAA GATCGGTTA-3' and 5'-CATGAAGAGGCGTATGCAGC-3' (set B; accession number AC009323); 5'-CTTTCTCGGACAAGATGGCA-3' and 5'-GTGTTGACAAGAACCTC GCT-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 AL049171 and 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, 2001) by PCR using the synthetic oligonucleotide sets A, B, C, D, E, F and 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, B and C as probes, and named *AtGolS1, 2* and *3,* respectively.

Analysis of DNA sequences

Plasmid DNA templates were prepared with the Automatic Plasmid Isolation System (model PI-100; KURABO, Osaka, Japan), and sequenced with a DNA sequencer (model 373A; ABI, San Jose, CA, USA). Nucleotide sequences and amino acid sequences were analyzed with the GeneWorks software system (IntelliGenetics, Inc., Mountain View, CA, USA) and the University of Wisconsin Genetics Computer Group (GCG) program.

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-labeled fragments using DIG RNA Labeling Kit (SP6/T7) (Roche Diagnostics, Indianapolis, IN, USA); the probes were PCR-amplified from the first-strand cDNAs by gene-specific primers. Following

hybridization, the filters were washed twice in 2 X SSC, 0.1% SDS for 5 min at room temperature and twice in 0.5 X 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, Indianapolis, IN, USA).

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

Expression of glutathione S-transferase (GST)-AtGolS genes 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*); and 5'-CGCGGA TCCATGGCACCTGAGATCAATACC-3' and 5'-CGCGGATCCGAGGCGTATGCAG CAACGAGC-3' (*AtGolS2*); and 5'-CGCGGATCCATGGCACCTGAGATGAACAACA AGTTG-3' and 5'-CGCGGATCCCTGGTGTTGACAAGAACCTCGCTC-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. This chimeric genes were expressed in *E. coli*. The procedures for the purification of the fusion protein have been previously described 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). 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 myo-inositol, 160 μg of BSA, and 0.017 mg of enzyme prepared as described above. The enzyme was preincubated 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 of ice-cold 100% ethanol and centrifuging (10000 X *g* for 15 min) in a microcentrifuge. The extract was dried up, and products were analyzed 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, crashed, added with 10 ml of 80% ethanol preheated to 80°C, and then boiled for 10 minutes at 90°C. Then the series of steps were repeated twice (a total of three rounds of these steps is performed). Next, sugars of the cell extracts were analyzed by HPLC as described above.

Construction of transgenic plants

To generate transgenic plants with sense *AtGolS* cDNA, I 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 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). I introduced these plasmids into wild-type *Arabidopsis* seedlings by *Agrobacterium*-mediated transformation. T_2 seeds were used for subsequent experiments.

Evaluation of drought tolerance

The transgenic plants were sown on germination medium (GM) containing 10 mg/l of hygromycin and were grown for 2 weeks for the selection as a trasgene introduction stocks. Growing plants were transplanted in the pot which equalized the quantity of the soil with 1000-fold diluted Hyponex™, and grown for 1 week to adapt in the soil. These total 3-week-old soil-grown plants exposed to drought stress by transferring vats without water for 2 weeks. After the drought treatment, these plants were rehydrated, and were left for 5 days.

Measurement of leaf transpiration rate

The transpiration rate of *Arabidopsis* plants was measured in fully expanded leaves with a portable photosynthesis system (model LI-6400, Li-Cor, Lincoln, NE, USA) under the following conditions: 100 μ mol/m^2/s, 350 ppm CO_2, 22°C, and 70% relative humidity.

Full-length cDNA microarray analysis

Full-length cDNA microarray analysis was carried out essentially as reported previously (Seki *et al.*, 2001). In this study, I used a cDNA microarray containing ca. 7000 *Arabidopsis* full-length cDNAs (Seki et al., unpublished) and drought-inducible genes, *RD* and *ERD* (Taji *et al.*, 1999). mRNAs from 35S-*AtGolS2* transgenic plants and wild-type control plants (transformed with the vector pBIG2113N) were used for preparation of Cy3-labeled and Cy5-labeled cDNA probes, respectively. These cDNA probes were mixed in equal amounts, and hybridized with the cDNA microarray.