Introduction

Sugars are important energy sources and structural components that act not only as metabolic substrates but also as signaling molecules at the level of gene expression. Expression of numerous genes is under metabolic control, and changes in metabolite concentration result in either induction or repression of these genes in both prokaryotes and eukaryotes (Newgard and McGarry 1995, Ronne 1995, Saier et al. 1995). In yeast, glucose represses the transcription of a large number of genes required for the utilization of alternative carbohydrates (Carlson 1999, Johnston 1999). In spite of sugar repression in plants being widely studied, knowledge of the regulatory mechanisms of both sugar sensing and responses remains limited compared with that for yeast, presumably due to more complex cellular metabolic pathways.

New insights into regulatory mechanisms of plant sugar sensing have emerged from recent advances in analysis of the Arabidopsis transcriptome. Several groups have analyzed the global expression profile of Arabidopsis genes in response to sugars, giving clues to understanding overall sugar responses (Koch 1996, Contento et al. 2004, Lee et al. 2004, Price et al. 2004, Thimm et al. 2004, Buchanan-Wollaston et al. 2005). In particular, a number of genes for carbohydrate metabolism, such as genes encoding glycosyl hydrolases, have been classified into >100 families based on amino acid sequence similarity. Their distinguishing features and representative members are described on the CAZy(ModO) server (Coutinho and Henrissat 1999). Rice α-amylases, a well-characterized class of glycosyl hydrolases induced by sugar deprivation, catalyze the hydrolysis of starch, which enables remobilization of sugars (Yu et al. 1991, Sheu et al. 1994, Sheu et al. 1996).

As mentioned above, transcriptomic analysis of Arabidopsis genes indicated that many genes for hydrolases assigned to cell wall modification increase in response to sugar starvation. Just as α-amylases produce sugars from starch, these cell wall glycosyl hydrolases may degrade cell walls to release sugars under sugar starvation conditions. However, cell wall polysaccharide-hydrolyzing enzymes have been mainly examined for their roles in fruit softening, seed germination and tissue development (Ross et al. 1994, Smith and Gross 2000, Martinez et al. 2004, Minic et al. 2004, Esteban et al. 2005, Hrubí et al. 2005).

In the present study, we characterized cell wall glycosyl hydrolases induced by sugar starvation to investigate the hypothesis that these cell wall glycosyl hydrolases can remobilize sugars.

Results

Genes encoding glycosyl hydrolases are induced by sugar starvation

The levels of transcription of three genes, At5g56870, At5g49360 and At3g60140, were analyzed by RNA blot
analysis under sugar starvation conditions. Mature leaves were detached and floated on water with and without sucrose in the dark. As shown in Fig. 1, mRNAs of At5g56870 and At5g49360 accumulated within 3 h and continued until at least 79 h after sugar starvation began. The At3g60140 transcript was detected 12 h after sugar starvation, and its level significantly increased until at least 79 h. Transcripts of At5g56870 and At5g49360 were already observed before treatments, and decreased with sucrose. It might suggest that leaves were in sugar-limited conditions inducing these two genes before treatment.

At5g56870, At5g49360 and At3g60140 encode proteins with 724, 774 and 577 amino acids, respectively (Fig. 2). They have domains characteristic for glycosyl hydrolase families 35, 3 and 1, respectively, the features of which are described on the CAZy server (Coutinho and Henrissat 1999). A prediction program for protein localization, PSORT, indicated that all three contain signal peptides for secretion and up to five potential N-glycosylation sites.

A BLAST search of the AGI transcript database at the Arabidopsis Information Resource (http://www.arabidopsis.org/blast/) revealed that these genes, which encode a putative β-galactosidase (At5g56870), β-xylosidase (At5g49360) and β-glucosidase (At3g60140), belong to Arabidopsis families containing 14, seven and 18 genes, respectively. Fig. 3A, B represents the phylogenetic tree of genes related to At5g56870 and At5g49360, constructed by aligning the amino acid sequences. Several genes showed a strong identity (43–80%) with these two identified genes. For the β-glucosidase (At3g60140), 18 genes were in the family, but the identities were not strong enough to consider any to be strong homologs.

In order to ensure that transcripts of identified genes were specifically induced by sugar starvation and not due to cross-hybridization of related genes, RNA blot analysis was performed. Although other genes such as At3g52840 and At5g64570 also responded to sugar starvation, transcripts for At5g56870 and At5g49360 were prominently induced by sugar starvation (Fig. 3C, D).

Fig. 2 Deduced amino acid sequences of the three glycosyl hydrolases. Deduced amino acid sequences of β-galactosidase (At5g56870) (A), β-xylosidase (At5g49360) (B) and β-glucosidase (At3g60140) (C). Putative signal peptides are indicated by a rectangle. Putative N-glycosylation sites are indicated by a double underline. Underlines indicate the domains of glycosyl hydrolase families 35, 3 and 1, respectively.
Degradation of cell wall following sugar starvation

β-D-xylosidase in young barley seedlings (Lee et al. 2003). At3g60140 is also called the din2 gene, which is up-regulated in response to darkness or senescence (Fujiki et al. 2001), while its physiological role remains unknown. Based on the homology search results, we inferred that the products of these three genes are secreted into the apoplastic space and function in cell wall degradation.

Secretion of the β-galactosidase following sugar starvation

In order to examine whether the protein secreted by sugar starvation is indeed associated with the accumulation of transcripts described above, cultured cells were chosen since secretory proteins could easily be recovered from the culture medium. As shown in Fig. 4A, transcription of the

Fig. 3 Gene families encoding β-galactosidases and β-xylosidases in Arabidopsis. Phylogenetic trees of the At5g56870 (β-galactosidase)- (A) and At5g49360 (β-xylosidase)- (B) related genes. The trees were constructed by alignment of the amino acid sequences using the cluster algorithms at http://taxonomy.zoology.gla.ac.uk/rod/treeview.html. The percentage indicates the amino acid identity with At5g56870 and At5g49360. Expression of At5g56870- (C) and At5g49360- (D) related genes. Detached leaves were incubated in the dark in the presence (+S) or absence of 1% sucrose (−S) up to 79 h and were collected at 0, 12, 36 or 79 h (lanes 1–7). Detached leaves incubated in the dark in the absence of sucrose for 72 h and then incubated for 7 h with 1% sucrose (−S/+S) were also used for experiments (lane 8). Total RNA (5 μg) extracted from detached leaves in each experiment was analyzed by RNA blot analysis.

Fig. 4 Induction of glycosyl hydrolases in cultured cells by sugar starvation. (A) Expression of three genes encoding glycosyl hydrolase. Seven-day-old cells were transferred to medium containing various concentrations of sucrose and incubated for 12, 24 or 36 h under darkness. An equal amount of RNA (10 μg) was loaded per lane and subjected to hybridization with the indicated probes. rRNA was visualized with ethidium bromide staining for the loading control. (B) Identification of secretion of the β-galactosidase protein. Cells were incubated in the medium with (+S) or without 3% sucrose (−S) for 72 h. Proteins in both culture media were compared by SDS–PAGE (left panel). An arrow indicates the β-galactosidase (At5g56870) identified by mass spectrometry analysis. Culture medium of cells incubated in the medium with (+S) or without 3% sucrose (−S) were recovered at the appropriate time and subjected to immunoblot analysis using an anti-β-galactosidase antibody (right panel).
three genes was tightly regulated by the sucrose concentration. Transcripts were detected within 12 h of sugar starvation, while 10 mM sucrose almost completely repressed their expression.

Proteins were prepared from culture medium of cells cultured with and without sucrose and compared by SDS-PAGE. Six bands which seemed to be specific to the culture medium without sucrose were subjected to mass spectrometry analysis. The data from the mass spectrometry analysis was further analyzed by database search (mascot search), resulting in identification of the proteins. All of them were secretory proteins having putative signal peptides (data not shown) and one of them was a β-galactosidase encoded by At5g56870. The products of At5g49360 and At3g60140 were not identified by this analysis probably due to the small amount of proteins or contamination with other proteins with similar molecular weight.

An antibody against the β-galactosidase was prepared using a recombinant protein produced in Escherichia coli. The β-galactosidase was clearly induced in culture medium depleted of sucrose, but not in the medium of cells cultured with sucrose (Fig. 4B). This experiment showed that at least the β-galactosidase encoded by At5g56870 was secreted under sugar starvation.

**Effects of metabolites on induction of the glycosyl hydrolase genes**

Transcription of the three glycosyl hydrolase genes was tightly regulated by sucrose in cultured cells as well as in intact plants. By taking advantage of suspension cells, the culture medium of which can be easily modified, the effect of various sugars on repression of these genes was examined. As shown in Fig. 5, glucose, galactose, fructose and xylose were as effective as sucrose in repressing these genes, while mannitol had no effect on repression, indicating that expression is regulated specifically, not osmotically. In addition, neither 2-deoxy-glucose nor 3-O-methyl-glucose repressed the gene expression, and pyruvate, which is derived from glycolysis and could be used as a carbon source, did not repress their expression.

**Induction of glycosyl hydrolases in the dark**

One of the most efficient stimuli that accelerate sugar starvation in nature is inhibition of photosynthesis. Thus, whole plants were kept in darkness for 24 h to induce natural sugar starvation. As shown in Fig. 6A, transcripts of the three hydrolase genes increased in all organs when plants were kept in prolonged darkness. We also examined the pattern of β-galactosidase (At5g56870) expression using transgenic plants harboring a chimeric gene consisting of the β-galactosidase promoter and β-glucuronidase (GUS) reporter gene. GUS staining was observed in rosette leaves and flowers, consistent with the results of RNA blot analysis when plants were placed in the dark for 24 h (Fig. 6B). In addition, when part of a rosette leaf was shaded to repress photosynthesis locally, GUS staining was strong in the covered region (Fig. 6B). In order to verify that β-galactosidase protein was indeed induced and secreted to cope with sugar starvation in planta, cell wall proteins were prepared from leaves. As shown in Fig. 6C, β-galactosidase protein started to accumulate after 12 h in darkness.

When detached leaves were floated on sucrose solution in the dark, β-galactosidase was not detectable. However, it clearly accumulated if sugar was not supplied (Fig. 6D). In similar conditions, the enzymatic activity of β-galactosidase was monitored by measuring the release of sugars from a cell wall polysaccharide, galactan. Hydrolyzing activity against galactan increased when sucrose was omitted. In contrast, its level was lower in the presence of sucrose (Fig. 6E).

**Cell wall degradation during sugar starvation**

Cell wall components were analyzed in detached leaves starved for sugar for 48 h, when β-galactosidase accumulation peaks in response to sugar starvation. Quantitative analysis of cell wall polysaccharides revealed that the levels of pectin, hemicellulose I (HC-I) and HC-II were clearly lower in sugar-starved leaves than in sugar-abundant leaves (Fig. 7A). In particular, the HC-I level decreased by approximately one-third under sugar-starved conditions. However, the amount of cellulose did not change significantly.
The monosaccharide content of each cell wall fraction was determined by gas–liquid chromatographic (GLC) analysis (Fig. 7B). Arabinose and xylose, major components of hemicellulose side chains, clearly decreased in sugar-starved leaves. A marked loss of galactose and glucose content, a component of pectin and hemicellulose, was also observed during sugar starvation. Rhamnose, fucose and mannose content was slightly lower in sugar-starved leaves.

Discussion

We previously identified genes induced by sugar starvation in Arabidopsis, including genes for a putative β-galactosidase (At5g56870) and β-xylosidase (At5g49360) (Lee et al. 2004). In the present study, we focused on these two genes and a putative β-glucosidase (At5g60140) reported as dark-inducible gene 2 (din2) (Fujiki et al. 2000), since the product of din2 has characteristics similar to those of the products of At5g56870 and At5g49360.

Although the functions of these three genes have not been reported, a homology search predicted that they are involved in modification of cell wall polysaccharides. Their products have characteristic glycosyl hydrolase domains and appear to catalyze the breakdown of cell wall sugar residues from cell wall polysaccharides. For instance, β-galactosidases, a widespread family of glycosyl hydrolases, are able to attack β-1,4-galactan bonds to produce galactose (Carey et al. 1995). β-D-Xylosidase can cleave xylan and arabinoxylan to produce xylose in stem tissues of Arabidopsis (Minic et al. 2004). The major β-glucosidase from germinated Nasturtium cotyledonary tissue seems to play a role in xyloglucan mobilization in vivo (Crombie et al. 1998). From these observations, we infer that these three enzymes play roles in the breakdown of cell wall polysaccharides. Since they are induced by sugar starvation, their physiological role in cell wall degradation is likely to be supplying a source of carbon. Although the direct substrates of these enzymes are not clear, several reports support our hypothesis.
Microbial cell wall-degrading enzymes play a role in penetration of plant pathogens and release of nutrients from the wall. It is well established that cell wall-degrading enzymes are repressed by catabolic repressors, such as glucose in fungi (Walton 1994). Moreover, starvation for carbon or nitrogen triggers very early expression and secretion of cell wall-degrading enzymes in order to yield these nutrients from target fungi (Donzelli and Harman 2001). In plants, the expression of genes coding for two α-galactosidase isoenzymes is induced by dark-induced senescence in primary foliage leaves of barley, suggesting the importance of this enzyme in leaf survival (Chrost et al. 2004; Roulin and Feller 2001) reported that activities of β-glucanase increased in the leaves of barley seedlings when they were incubated under dark conditions. They suggested that glucose was released from (1→3, 1→4)-β-D-glucan, and that released glucose was utilized as an energy source to sustain respiration and other metabolic processes in darkness.

Thimm et al. (2004) have analyzed the detailed global expression profile of Arabidopsis genes in response to sugar depletion; although many genes required for modification of cell walls were induced upon prolonged exposure to darkness, they suggested that changes in transcripts did not necessarily affect protein levels. Thus, it is difficult to interpret a physiological phenomenon just from the analysis of transcripts. In the present study, we detected accumulation of β-galactosidase protein during sugar starvation, showing more direct evidence to support our hypothesis.

Our hypothesis that cell wall polysaccharides are degraded in association with sugar starvation was directly tested by measuring the contents of cell wall poly- and monosaccharides. Galactose can be found in abundance in both hemicelluloses and pectins (Brett and Waldron 1996), and mannose, xylose, arabinose, galactose and glucose are typical components of hemicelluloses such as xylan and xyloglucan (Dey and Brinson 1984). Thus, it is likely that the reduction of galactose, xylose and glucose in both fractions under sugar starvation is due to the specific release of these sugar residues from various cell wall polysaccharides such as pectic galactan, xyloglucan and arabinoxylan by glycosyl hydrolases. This idea was supported by the finding that the polysaccharide hydrolyzing activity against galactan increased in association with depletion of sugars. We also observed similar activity against xylan (data not shown). It should be mentioned that accumulation profiles of β-galactosidase proteins and the time course of glycosyl hydrolase activity are not necessarily correlated. We consider that our measurement might include activities of other glycosyl hydrolases that may be also induced by sugar starvation. Considering our findings showing that cultured cells grow utilizing various monosaccharides (data not shown), these sugar residues could be transported into the cytoplasm from the apoplast and then used as a carbon source to cope with sugar starvation. Consistent with our idea, an increase in transcripts for exo-hydrolases dependent on sugar starvation was also reported (Buchanan-Wollaston et al. 2005).
The glucose analogs 2-deoxy-glucose and mannose are phosphorylated by hexokinase, whereas 3-O-methyl-glucose is transported into the cell but not phosphorylated (Jang et al. 1997, Smekens 2000, Moore et al. 2003). Our results suggest that the expression of at least At1g56870 and At3g60140 is regulated by metabolic hexose, lying upstream of pyruvate, as a flux sensor for sugar repression rather than a hexokinase-dependent pathway. This result is different from the mechanism controlling gene expression in response to sugar starvation during dark-induced senescence of Arabidopsis, which involves hexokinase as a sugar sensor (Fujiki et al. 2000, Fujiki et al. 2001). More studies will be needed to understand the signal transduction pathway.

In nature, sugar starvation is often induced by inhibition of photosynthesis. Our results indicate that three glycosyl hydrolase genes were highly induced and inhibition of photosynthesis. Our results indicate that the pathway.

medium of 3-day-old cell culture was filtrated with a nylon screen to remove cells. Proteins were recovered by addition of ammonium sulfate (70% saturation) to the medium and subsequent centrifugation (40,000×g for 20 min). Precipitates were washed with 80% acetone and dissolved in rehydration buffer {7 M urea, 2 M thiourea, 4% CHAPS (3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate)}. Proteins were separated by SDS-PAGE and stained with Coomassie brilliant blue R-250. Major bands were excised and in-gel digested with trypsin according to Ohta et al. (2002). After the reduction and alkylation treatments, proteins in the gel slices were digested with 12.5 μg ml⁻¹ of modified trypsin (Roche, Indianapolis, IN, USA) at 37°C for 16 h. Digested peptides were extracted with formic acid and acetonitrile. Peptide mixtures were separated and analyzed using an LC MAGIC 2002 (Michrom BioResources, Auburn, CA, USA), which was connected directly to an electrospray ion-trapping tandem mass spectrometer LCQ-Advantage (Thermo Electron, San Jose, CA, USA; Ohta et al. 2002). Mass spectrum data generated by the liquid chromatography–tandem mass spectrometry (LC–MS/MS) were used to search the NCBI non-redundant protein database with Mascot MS/MS Ion Search software (Matrix Science, Boston, MA, USA).

Production of recombinant β-galactosidase and its antibody
cDNA encoding the mature β-galactosidase protein was amplified by PCR and cloned into a pBAD/D-TOPO® vector in-frame with HP-thioredoxin and a C-terminal peptide containing the V5 epitope and a polyhistidine (6×His) tag. Purification of recombinant β-galactosidase (At1g56870) protein produced in E. coli and antibody production were conducted in the Medical & Biological Laboratories MBL Co., Ltd (Nagano, Japan).

Immunoblot analysis
Leaf samples were homogenized in 50 mM Tris–HCl (pH 7.5) supplemented with 100 mM NaCl, 0.05% Tween-20, 1 mM EDTA and 250 μM phenylmethylsulfonyl fluoride (PMSF). The homogenate was centrifuged at 20,000×g for 10 min. Soluble proteins in the supernatant and proteins in the culture medium, prepared as described above, were separated in a 10% SDS–polyacrylamide gel. Proteins were further electrotransferred to a polyvinylidene difluoride membrane. The membrane was probed with the primary anti-β-galactosidase (At1g56870) antibody. Signals were detected with horseradish peroxidase (HRP)-conjugated secondary antibodies and ECL (enhance chemiluminescence) Western blotting detection reagent (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Cell wall protein extraction
A 2 g aliquot of leaf tissue was homogenized with 3 ml of 20 mM sodium acetate buffer, pH 4.5, 1 mM EDTA. The cell wall fractions were collected by filtration through a nylon screen (80 μm mesh), and washed twice with the same buffer. Proteins of cell wall fractions were extracted for 2–3 h in 1 ml of

Materials and Methods

Plant material and treatments
An Arabidopsis thaliana Col-0 cell suspension derived from roots of Arabidopsis ecotype Columbia (Mathur et al. 1998) was cultured in 50 ml of culture medium (MS with 3% sucrose, 1 mg l⁻¹ of 2,4-dichlorophenoxyacetic acid and 1× B5 vitamins, including 4 mg l⁻¹ pyridoxine HCl, 4 mg l⁻¹ nicotinic acid, 40 mg l⁻¹ thiamine-HCl and 400 mg l⁻¹ myo-inositol, pH 5.8) by shaking on a rotary shaker (120 r.p.m.) at 25°C in the dark. To exchange medium, 7-day-old subcultured cells were collected on a filter paper using vacuum filtration and were resuspended in 50 ml of fresh culture medium with or without sugars.

Leaves of Arabidopsis (ecotype Columbia) grown at 22°C with a 16 h photoperiod for 3 weeks were cut in the middle part of the petiole. The detached leaves were floated on water or 1% sucrose solution.

Plants grown in soil were shaded by covering with black boxes for 3 d and harvested for analysis. In order to shade a part of a leaf, it was covered with a piece of aluminum foil for 1 d.

Database analysis
Nucleotide and deduced amino acid sequence comparisons against databases were carried out using BLAST searches. Signal sequence predictions were conducted using the interactive web site PSORT (Nakai and Horton 1999).

RNA gel blot analysis
RNA isolation and RNA gel blot analysis from suspension-cultured cells and leaves were performed as described (Lee et al. 2004).

Identification of proteins in culture medium by mass spectrometry
Medium of 3-day-old cell culture was filtrated with a nylon screen to remove cells. Proteins were recovered by addition of ammonium sulfate (70% saturation) to the medium and subsequent centrifugation (40,000×g for 20 min). Precipitates were washed with 80% acetone and dissolved in rehydration buffer {7 M urea, 2 M thiourea, 4% CHAPS (3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate)}. Proteins were separated by SDS-PAGE and stained with Coomassie brilliant blue R-250. Major bands were excised and in-gel digested with trypsin according to Ohta et al. (2002). After the reduction and alkylation treatments, proteins in the gel slices were digested with 12.5 μg ml⁻¹ of modified trypsin (Roche, Indianapolis, IN, USA) at 37°C for 16 h. Digested peptides were extracted with formic acid and acetonitrile. Peptide mixtures were separated and analyzed using an LC MAGIC 2002 (Michrom BioResources, Auburn, CA, USA), which was connected directly to an electrospray ion-trapping tandem mass spectrometer LCQ-Advantage (Thermo Electron, San Jose, CA, USA; Ohta et al. 2002). Mass spectrum data generated by the liquid chromatography–tandem mass spectrometry (LC–MS/MS) were used to search the NCBI non-redundant protein database with Mascot MS/MS Ion Search software (Matrix Science, Boston, MA, USA).
20 mM HEPES, pH 6.8, 1 M NaCl, 2 mM EDTA, 3 mM sodium metabisulfite at 4°C.

Assay of glycosyl hydrolase activity

The plant material (~1 g FW) was homogenized in liquid nitrogen and added to 1 ml of an extraction buffer (10 mM sodium phosphate buffer, pH 7.0). The homogenate was collected by centrifugation at 3,000 x g for 10 min to obtain the cell wall fraction. The pellet was washed twice with the same buffer and then resuspended in 500 μl of 10 mM sodium phosphate buffer (pH 6.0) containing 3 M NaCl. Each sample was incubated at 4°C for 24 h and recovered by centrifugation at 3,000 x g for 10 min. This protein fraction was assayed with galactan (ex. Lupin, Gal: Ara: Rha: Xyl: GalUA = 91:2:1.7:0.3:5 from Megazyme, Wicklow, Ireland). A 100 mg aliquot of substrate was dissolute in 10 ml of 10 mM sodium phosphate buffer (pH 5.0). The mixtures consisting of 50 μl of sample and 50 μl of substrate were incubated at 30°C for 30 min. Enzyme activity was measured by monitoring the increase of reducing sugars following the 3-methyl-2-benzothiazolinonehydrazone method (Anthon and Barrett 2002).

Determination of cell wall carbohydrate contents

Cell wall polysaccharides were fractionated by the method of Hoson et al. (1995). Methanol-boiled samples were rehydrated with water and homogenized. The homogenate was washed sequentially with water, acetone, a methanol:chloroform mixture (1:1, v/v) and ethanol, and treated with 2 μM-1 porcine pancreatic 8-amylase (EC 3.2.1.1; type I-A; Sigma, St Louis, MO, USA) in 50 mM sodium acetate buffer (pH 6.5) at 37°C for 3 h. After amylase treatment, the pectin fraction was extracted with 50 mM EDTA at 95°C, and HC-I and HC-II were successively extracted with 4 and 24% (w/v) KOH, respectively, containing 0.02% NaBH4 at 25°C. The resulting hemicellulose fractions were neutralized with acetic acid and dialyzed against water. The cellulose fraction was washed with 0.03 M acetic acid and ethanol, dried at 40°C, dissolved in 72% (v/v) sulfuric acid for 1 h at 25°C, and then diluted with a 29-fold volume of water. The total sugar content in each fraction was determined by the phenol-sulfuric acid method (Dubois et al. 1956) and expressed as glucose equivalents.

Gas-liquid chromatography

The neutral sugar composition of pectic and hemicellulosic polysaccharides was determined by the method of Albersheim et al. (1967). The samples were lyophilized, and then hydrolyzed with 2 M trifluoroacetic acid for 1 h at 121°C. After the trifluoroacetic acid had been removed by evaporation under a stream of air at 50°C, the sugars were reduced with sodium borohydride in 1 M ammonia, and acetylated with acetic anhydride for 3 h at 121°C. The amounts of the acetylated alditois derived from neutral sugars were analyzed by GLC using an alditol acetate derivative with myo-inositol as the internal standard.

Transgenic plants and GUS staining

Approximately 1.5 kb of the 5′-upstream region from the start codon for b-galactosidase was amplified by PCR from Arabidopsis genomic DNA and cloned into the pGEM-T easy vector. The amplified promoter was digested with BamHI and SalI, and cloned into the pBI101 binary vector containing the GUS reporter gene. The chimeric gene construct was introduced into Arabidopsis by Agrobacterium-mediated transformation (Clough and Bent 1998). Transgenic plants were selected for kanamycin resistance, and the presence of the transgene was confirmed by PCR. GUS expression profiles were determined according to the procedure described by Jefferson et al. (1987).

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