	Molecular characterization of sugar-responsive genes in *Arabidopsis thaliana*		

In plants, sugars are major substrates for synthesis of complex carbohydrates such as starch and cell wall, as well as respiratory substrates. In addition, sugars play an important role as signaling molecules modulating expression of various genes involved in diverse physiological processes. For example, genes encoding photosynthetic enzymes are repressed by sugar supply since photosynthesis is not necessary under such a condition. Genes for starch breakdown are induced by sugar starvation to generate more sugars. However, the mechanism by which these genes are regulated by sugars remains to be elucidated. In this study, I focused on identification and characterization of genes that are regulated by sugars using a model plant, *Arabidopsis thaliana*.

In the chapter I, I screened genes whose expression was regulated by sucrose using cDNA macro-arrays, and confirmed thirty-six genes to be regulated by sucrose supply in detached leaves by RNA blot analysis. Eleven of them encoding proteins for amino acid metabolism and carbohydrate metabolism were repressed by sugars. The remaining genes induced by sugar supply were for protein synthesis including ribosomal proteins and elongation factors.

In the chapter II, I characterized genes encoding putative β-galactosidase, β-xylosidase and β-glucosidase that were induced by sugar depletion. Since those proteins showed a high similarity with cell wall-degrading enzymes, it was conceivable that those hydrolases were induced to breakdown cell wall materials producing carbon source under sugar-starved condition.

In fact, activities of β-galactosidase, β-xylosidase and β-glucosidase increased in culture medium of suspension cells. Secretion of β-galactosidase into culture medium was also confirmed using antibody. In addition, suspension cells grew with galactose, xylose and glucose as carbon source as well as with sucrose. These sugars repressed induction of genes for the putative cell wall degrading enzymes, suggesting that plant cells released those sugars from cell wall and consumed them. This was directly confirmed by measurement of cell wall polysaccharides contents showing a marked decrease of pectin and hemicellulose I associating with sugar starvation. These results supported the idea that one of functions of cell wall is to serve as 'storage of carbon source'. RNA blot analysis and the experiment using GUS reporter gene indicated that genes for those cell wall degrading enzymes were induced in most of organs when plants were kept in dark for one day. It was also suggested that induction of these genes was not mediated by hexokinase-dependent signaling pathway that often plays a role in sugar signaling.

In the chapter III, I attempted to identify *cis*-element of sugar responses using a typical sugar-induced gene. I analyzed the promoter of gene encoding a protein kinase which was induced by sugar as well as cold and salt stresses. Promoter dissection suggested that the element for sugar induction was different from those for other stresses.

Molecular Characterization of Sugar-Responsive Genes

in *Arabidopsis thaliana*

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INTRODUCTION

Sugars in plants

Sugars are important energy sources and structural components in living cells. In plants, sucrose and starch are the end products of photosynthesis. Sucrose is generated in leaf cytosol, and transported through phloem to sink tissues, where it is metabolized or stored in the vacuole (Taiz and Zeiger, 1991). During night, sucrose is derived from starch, which is produced and stored in chloroplasts during daytime. In contrast, starch is synthesized from sugar and stored in amyloplasts in non-photosynthetic cells (Fernie *et al*., 2002).

Sugars play an important role in growth and development throughout the life cycle of plants, through germination to flowering or senescence (Steeves and Sussex, 1989; Brussian and Tobin, 1992; Graham *et al*., 1992; Bernier *et al*., 1993; Sheen, 1994; Thomas and Rodriguez, 1994; Dangel *et al*., 1995). In this process, sugars act as regulatory molecule controlling metabolism, cell cycle, development, and gene expression. In the past decade, sugars have been shown to be physiological signals, repressing or activating expression of genes involved in photosynthesis, glycolytic metabolism, respiration, starch synthesis and degradation, nitrogen metabolism, pathogen defense, sounding response, cell cycle regulation, pigmentation, and senescence (Chen *et al*., 1994; Knight and Gray, 1994; Lam *et al*., 1994;

Sheen, 1994; Herbers *et al*., 1995; Mita *et al*., 1995; Reynolds and Smith, 1995).

Cellular metabolism and sugars

Plant growth depends on the partitioning of assimilated carbon between photosynthetic sources such as mature leaves, and photosynthetically less active or inactive sink tissues such as roots, flowers and fruits (Farrar and Williams, 1991). Environmental changes also affect biochemical reactions, by disturbing the balance of metabolites within cells. An example is sugar starvation, which changes substantial physiological and biochemical processes to keep respiration and other essential metabolic processes. In sugar-starved cultured cells, a decrease in enzymatic activities related to sugar metabolism and respiration (Journet *et al*., 1986; Brouquisse *et al*., 1991), nitrate reduction and assimilation (Brouquisse *et al*., 1992), and protein synthesis (Tassi *et al*., 1992) is obvious. Such decreased activities were considered to be associated with protection of cells against nutrient stress by switching off biosynthesis not to consume energy. In contrast, enzymatic activities related to catabolism of fatty acids (Dieuaide *et al*., 1992), amino acids (Brouquisse *et al*., 1992), and proteins (Tassi *et al*., 1992) increase. Thus, plants possess complicated mechanisms to recycle metabolites during growth, developmental remodeling, and nutrient-limiting conditions (Callis, 1995; Vierstra, 1996). Especially protein degradation is critical for nitrogen and carbon economy. Protein turnover in vacuole is thought to play a prominent role under condition when rapid remobilization and

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resorption of nutrients is needed. In spite of these metabolic changes, carbohydrate starvation ultimately results in cells death after irreversible damages (Brouquisse *et al*., 1991; Chen *et al*., 1994). Plant cells have intracellular pool of carbohydrate and autophagic process to survive for a while without external supply of organic carbon. This occurs under nutrient deprivation, which is induced by environmental stresses and developmental organ remodeling such as senescence (Marty, 1999; Kim and Klionsky, 2000). A mechanism which regulates metabolic processes during sugar starvation and senescence was suggested to be common (Noodén, 1988).

Genes regulated by sugar starvation

A feedback repression of gene expression by excess sugar provides an additional mechanism for maintaining an economical balance between supply (source) and demand (sink) for carbohydrate allocation and utilization among tissues and organs (Sheen, 1994; Thomas and Rodriguez, 1994; Yu *et al*., 1996). In other words, sugar starvation initiates changes in cellular processes to recycle cellular constituents, and dramatically changes the pattern of gene expression (Koch, 1996). In general, carbohydrate depletion up-regulates genes for photosynthesis, remobilization and export of carbohydrate. For example, sugar depletion induced the expression of photosynthetic genes in maize mesophyll protoplasts (Sheen, 1990), chlorophyll *a/b*-binding protein gene in *Arabidopsis* (Oswald *et al*., 2001),

malate synthase, and isocitrate lyase genes in cucumber (Graham *et al*., 1994). Abundant

sugar exerts opposite effects through a combination of gene repression and induction (Koch

1996). Transcript level of elongation factor 2 (eEF2) decreased in response to carbohydrates

starvation in sugar beet (Vogel *et al*., 1999). Increase of ammonium-transporter transcripts

was related to carbohydrate abundance in roots of field pea and rutabaga (Kubik-Dobosz *et al*.,

2001). Thus, sugar repression of gene expression appears to be one of major control

mechanisms, which regulate energy homeostasis and carbohydrate distribution in plants.

Sugar sensing and signaling in plants

Despite the fundamental role of sugars, controlling mechanism of sugar sensing and gene

regulation is not fully understood. Using mutants which show physiologically abnormal

response by exogenously applied sugars, and feeding experiments with non-metabolizable

sugar analogs, sugar transporters and hexokinase (HXK) were suggested to be involved in

perception of sugar signal (Lalonde *et al*., 1999; Sheen *et al*., 1999; Smeekens, 2000).

Hexokinase was proposed as a glucose sensor, which modulates gene expression and multiple

plant hormone-signaling pathways (Sheen *et al*., 1999; Smeekens, 2000). From biochemical

and genetic experiments, the hypothesis that hexokinase is a glucose sensor was confirmed

(Moore *et al*., 2003).

Glucose analogs such as 2-deoxy-glucose and mannose are phosphorylated by hexokinase,

but not further metabolized (Sheen, 1994; Jang and Sheen, 1994; Matin *et al*., 1997; Koch, 1996). 3-*O*-methyl-glucose is transported into the cell but not phosphorylated by hexokinase (Jang and Sheen, 1997; Lalonde *et al*., 1999; Smeekens, 2000; Rolland *et al*., 2002). These compounds have been widely used to investigate sugar sensing. (Graham *et al*., 1994; Jang and Sheen, 1994; Godt *et al*., 1995; Martin *et al*., 1997; Fujiki *et al*., 2000; Ichimuta *et al*., 2000; Ho *et al*., 2001; Oesterhelt and Gross, 2002), and results showed that, although phosphorylation of hexose was required for signal perception, its further metabolization was not required in the hexokinase-dependent pathway. Beside sucrose, trehalose, and other hexokinase-independent sugar sensing mechanism was recently proposed (Goddijn and Smeekens, 1998; Lalonde *et al*., 1999; Smeekens, 2000). They suggested that sugars such as sucrose and trehalose, which are not hexose, might directly serve as the signal.

Cell wall in plants

Cell wall in higher plants has complicated structures, which are modified during cell expansion, secondary thickening, and changing environmental condition. Cell wall is generally considered to mechanically support cell, and to serve as a source of signaling molecules (Miller *et al*., 1997). Cell wall is mostly composed of polysaccharides (cellulose, hemicelluloses, and pectins), proteins, and aromatic compounds (lignins and other wall-linked phenolic acids). There are two types of cell wall deposition. The primary cell wall determines

cell shape and size during growth and development. The secondary cell wall, which is

deposited in fully expanded and specialized cells, contributes to thickening of cell wall by

accumulating polysaccharides and/or aromatic components. Cellulose represents the major

constituent of cell wall polysaccharides and is constructed by linear polymer of β-1,4-linked

D-glucose. Cellulose polymer has ordered structure, and its primary function is to add the

rigidity to cell wall. Hemicelluloses are heterogenous polysaccharides and the second most

abundant organic components in cell wall. The predominant hemicellulose of most cell wall is

xyloglucan, which consists of a branched (1->4)-β-D-glucan backbone containing substitution

at the O-6-position with short side-chains containing xylose, galactose and, often, a terminal

fucose (McNeil *et al*., 1984). Dynamic remodeling of xyloglucan crossing between cellulose

microfibril is believed to be important for cell wall expansion. In contrast, little information is

available on biological function of hemicellulosic fraction. Glucuronoarabinoxylan and other

hemicellulosic components are synthesized in the Golgi apparatus and exported to cell wall

(Bolwell, 2000; Bolwell and Northcote, 1983). Glucuronoarabinoxylan is an acid

polysaccharide consisting of a backbone of (1->4)-β-linked D-xylosyl residues. About 25% of

the xylosyl residues are substituted at the O-2 position with arabinosyl, glucosyluronic acid,

or 4-*O*-methyl glucosyluronic acid residues (Darvill *et al*., 1980). Pectic polysaccharides are

usually classified into homogalacturonans, rhamnogalacturonan I (RG-I) and

rhamnogalacturonan II (RG-II), which are consisted of a backbone of α-1,4-linked

D-galacturonic acid residues, with long side chains of arabinans and arabinogalactans (Lau *et al*., 1985). Hemicellulose and pectin polysaccharides, as well as aromatic polymer lignin, interact with cellulose fibrils, creating a rigid structure, which strengths cell wall. They also form covalent cross-links, resulting in limiting cell growth and reducing cell wall biodegradability.

Degradation of plant cell wall polysaccharides

Changes of cell wall structure occur not only during development but also in response to environmental condition. These changes accompany with induction of hydrolytic and oxidative enzymes. A mutant, *kor,* is defective a β-endoglucanase involved in cellulose metabolism (Sato *et al*., 2001), and showed impaired hypocotyls elongation in darkness (Nicol *et al*., 1998). The remodeling of xyloglucan catalized by xyloglucan endotransglycosylase is supposed to play roles in cell wall of growing tissue (Edelmann and Fry, 1992; Nishitani and Tominaga, 1992; Talbott and Ray, 1992; Nishitani, 1995; Xu *et al*., 1996) or during cell expansion (Fry, 1989; Smith and Fry, 1991; Fry *et al*., 1992). Ripening of fruits is considered to be the result of cell wall degradation catalyzed by mainly hydrolytic enzymes (Huber, 1983; Brady, 1987). Activities of β-D-xylosidase, α-D-mannosidase, β-D-galactosidase and β-D-glucosidase, were monitored during growth and ripening of fleshy pericarp of wild plums, peaches and apricots (Bouranis and Niavis, 1992). However, function

of those enzymes in normal tissues has not been clarified yet.

Aim of this study

As mentioned above, the importance of sugars have long been recognized not only in energy source, but also in cellular signaling molecule. In addition, they are components of polysaccharides, which constitute cell architecture. However, knowledge on the molecular mechanism, in which these processes are temporarily and specially well controlled, is limited. In order to shed light on these questions, I thought that global identification of sugar-responsive genes is indispensable.

Accordingly, I initiated this study first by identifying genes that were regulated by sugars thorough transcriptomic analysis, and found many genes, which were involved in protein synthesis, amino acid metabolism and carbohydrate catabolism. Among them, I selected particular genes that are presumably involved in degradation of cell wall components under sugar depletion, since their characterization is the first step for understanding function of sugars in metabolism of cell wall polysaccharides.

Here I describe properties of these genes and their products, showing structure, temporal and spatial expression pattern, activity and enzyme protein levels, and quantification of cell wall-derived polysaccharides. I also discuss that plants may reutilize carbon sources for energy source, which would sustain respiration and other metabolic processes under sugar depletion. Through these analyses, I conclude that plants possess a well-turned nutrient

recycling system, which is indispensable for them to maintain their life.

CHAPTER 1

SCREEING OF SUGAR-RESPONSIVE GENES

As described in Introduction, sugars act as signaling molecules regulating expression of sets of genes. In principal, sugars induce genes for utilization of sugars and repressed genes for production of sugars. For instance, sucrose induces genes for starch synthesis and represses photosynthetic genes such as Rubisco (Krapp *et al*., 1993). Genes for starch breakdown are also repressed by sugars, or induced by sugar starvation (Yu *et al*., 1991). Since sugars are abundant compounds in cellular metabolism, it was conceivable that they affect various phenomena that had not been discovered as yet. For example, high concentration of sucrose in medium was found to prevent seed germination of *A.thaliana*. Taking advantage of this phenomenon, *Arabidopsis* mutants were isolated, and provided important information for understanding of sugar signaling pathways (Léon and Sheen, 2003). In addition to such forward genetics, determination of complete genome of *Arabidopsis* made the reverse genetic approach common, in which comprehensive analysis of transcriptome became possible using DNA arrays. In this chapter, I focused on identification of genes whose expression profiles were affected by exogenous sugar supply.

RESULTS

Macro-arrays screening identifies sugar-responsive genes

I used cDNA macro-arrays containing approximately 13,000 *Arabidopsis* EST to screen genes whose transcript levels changed in response to sugar status. Probe cDNAs for differential hybidization were prepared from hydroponically cultured seedlings which were subjected to sucrose depletion for 79 h, or after return to sucrose-rich medium for 7 h following 72 h sucrose depletion. Hybridization was performed in duplicate for each sample to obtain reproducibility. The signal was normalized to calculate the intensity ratio between the two probes. Initial screening yielded 184 genes with either >3- or <0.3 fold change in signal ratios (Fig. 1-1a). Subsequently, duplicated sub-arrays were prepared, each containing 192 genes: 184 initially identified and 8 positive control genes. Analysis of these sub-arrays revealed transcript levels of 73 genes to show a 1.5-fold increase or decrease in comparison with the controls (Fig. 1-1b).

Transcript accumulation was regulated by exogenous sugar supply

In order to confirm the result of sub-array analysis, expression profile of the 73 genes detected in the arrays were further examined by RNA blot analysis. Seedlings grown in culture medium containing sucrose or detached leaves of 25-day-old plants were transferred to a fresh medium without sucrose and incubated for 12, 36 and 79 h in darkness, total RNA

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was isolated. Northern hybridization identified 24 genes whose transcripts were repressed by sucrose depletion (Fig. 1-2). About half of them were found to encode ribosomal proteins. The remaining included genes for proteins involved in protein synthesis, such as elongation factors and molecular chaperons, for proteins involved in amino acids metabolism, secondary metabolism, energy metabolism. Genes with unknown function was also identified. Annotation of these genes is listed in Table 1-1.

I also identified 11 genes whose transcripts were induced by sucrose depletion, and the accumulation pattern of their transcripts is shown in Fig. 1-3. Classification of these genes was presented in Table 1-2. Since I was interested in genes functioning under sugar starvation, I further characterized only those up-regulated by sucrose depletion in the following study.

Sugar contents correlated with transcript accumulation

When developed leaf samples detached from 25-day-old-plants were supplied with 30 mM sucrose, sucrose and glucose increased over 5-fold higher than the initial levels within 79 h (Fig. 1-4a). In contrast, when samples were deprived of sucrose, the levels of sucrose and glucose declined. When samples were deprived of sucrose for 72 h and then supplied with 30 mM sucrose for 7 h, both sucrose and glucose retrieved nearly 50% of those in sugar-supplied samples.

The time course of transcript accumulation of 11 up-regulated genes was examined in

sucrose-supplied and sucrose-deprived detached leaves (Fig. 1-4b). Whereas all of their transcripts were reduced in sucrose-supplied samples, they rapidly accumulated upon sucrose deprivation. Notably, sucrose-induced transcripts declined within 7 h after sucrose was re-supplied (Fig. 1-4b). These patterns appeared to be shared in common, although the initial levels of some transcripts were already distinct before treatments. This might be due to the standard growth condition, under which plants were possibly exposed to a slight sugar deficiency, to which some genes sensitively responded.

Shading induced transcripts of sugar-responsible genes

When whole plants were shaded, both sucrose and glucose contents in rosette leaves significantly decreased by up to 25 % of the initial level after 3 d (Fig. 1-5a). The decline was more distinct for glucose than for sucrose, showing a 70 % reduction after 1 d treatment. The transcripts of nine genes in attached leaves were highly induced within 1 d of transfer to the dark and the level was maintained for up to 3 d (Fig. 1-5b). We used *Sen1*, whose expression is generally known to be associated with artificially induced senescence (Shimada *et al.*, 1998; Shimizu *et al.*, 2001), as a control.

Senescence affected transcript accumulation

Detached leaves are known to senesce, showing visible yellowing (Fig. 1-6a). Using detached rosette leaves, which were allowed to age, variation in sugar content and transcript

levels of the isolated genes were examined. The experimental system was set up with a Columbia ecotype, in which rosette leaves fully expand at approximately day 25 after germination and inflorescences and flowers develop at day 35. At day 50, rosette leaves lose chlorophyll, and at day 70 the plants reach mature senescence. Sucrose and glucose, which accumulated until day 25, rapidly decreased during the next 10 d (Fig. 1-6a). However, amounts of both sugars increased thereafter, reaching at day 70 almost the same level as at day 25. This may indicate the breakdown of accumulated starch or cellular components.

Using this system, I examined transcript levels of my genes by RNA blot hybridization. Unexpectedly, their response was variable (Fig. 1-6b), first showing not all transcripts to be induced, and then induction becoming obvious after day 50. Transcripts for *At2g13360* and *At5g43060* were present in non-senescent leaves at day 25, and persisted at a relatively constant level throughout the aging period. Furthermore, *At5g56870* was not induced by leaf senescence. These results suggested that expression system of identified genes differs between artificial senescence induced by darkness and sugar depletion, and age-related natural senescence.

DISCUSSION

This chapter documents identification and expression profile of sugar-responsive genes from *Arabidopsis*. Screening with a macro-array and subsequent confirmation with RNA blot

hybridization identified 35 genes, whose transcripts responded to sugar status. Among them 24 and 11 transcripts were up- and down-regulated by sugars, respectively. However, I will not discuss further on up-regulated genes in this Chapter, since most of them were associated with an increase of protein synthesis due to sugar supply.

In plants, sugar depletion seriously affects cellular metabolism, and activates metabolic pathways to produce energy for remobilization of sugars through catabolism of proteins, amino acids and lipids (Tassi et al., 1992; Moriyasu and Ohsumi, 1996). These procedures are accompanied by transcriptional activation of relevant genes, including those associated with photosynthesis, reserve remobilization, and carbohydrate metabolism (Koch, 1996). They are called "famine genes", and are generally regulated by sugar molecules (Koch, 1996). Despite the importance, clear examples of famine genes known to date are few, probably numbering less than thirty. The present screening added to the list at least 8 new members, which have not so far been known to be sugar-responsive. In this context, their characterization is important to expand our knowledge of physiological responses of plants to sugar depletion.

Under dark and sugar-deficient conditions, starch is first subjected to breakdown followed by lipid and protein breakdown, the products being utilized as carbon sources. Expression of genes involved in carbon and nitrogen metabolism is altered in a coordinated fashion. For example, increase in endogenous sugars through breakdown of proteins and storage lipids likely activates expression of genes encoding asparagine synthetase, which is associated with

transport of nitrogen to developing tissues and storage organs (Lam *et al*., 1994; Sakurai *et al*., 1996). My results are consistent with this view, showing up-regulation of alanine aminotransferase, which synthesizes storage nitrogen amino acids such as alanine. Conceivably they play specific roles in maintenance of carbon and nitrogen balance, and transport and reallocation of nitrogen. This idea is further consistent with up-regulation of proline oxidase and cysteine proteases, both of which may be involved in nitrogen remobilization. For example, degradation of proline is observed when plants are maintained in the dark, and expression of the proline oxidase gene is down-regulated by sugars (Sanada *et al*., 1995; Hayashi *et al*., 2000; Hellmann *et al*., 2000). Cysteine proteases have been suggested to function in degradation of cellular materials including proteins and nucleic acids in senescing tissues (Drake *et al*., 1996; Solomon *et al*., 1999; Yamada *et al*., 2001). Taking the available information together, it can be concluded that co-operation among asparagine synthetase, alanine aminotransferase, proline oxidase and cysteine proteases may be important for nitrogen transport and reallocation after sugar depletion.

Transcriptional induction of genes involved in carbohydrate metabolism was here found to be notable during sugar depletion. Among four genes identified, two encoded a seed imbibition protein and its homolog. It was previously pointed out that seed imbibition proteins in dark-adapted and senescent leaves are probably related to metabolism of raffinose family oligosaccharides (Fujiki *et al.,* 2001), major carbohydrates in plants that appear to function as

carbon stores in seeds. They are also transported to leaves to cope with cold and/or freezing stress as well as winter conditions (Wiemken and Ineichen, 1993; Keller and Matile, 1985**).** A similar induction profile under sugar depletion suggests a common function of raffinose family oligosaccharides. The other two genes encode β-galactosidase and β-xylosidase, which might be involved in breakdown of cell wall components. The strong induction of their transcripts observed in leaves after sugar depletion suggests that they function in the release of galactose and xylose from cell wall macromolecules to produce sugars. Such a phenomenon has not been previously observed, and further analysis may reveal novel features of molecular mechanisms underlying recycling of cellular components. I focused on these genes in the next Chapter. In addition to genes involved in nitrogen and carbon recycling, three miscellaneous examples were here found, which probably function in membrane transport, sulfur metabolism and phytohormone-associated metabolism. Their relationship to sugar depletion remained to be elucidated.

Finally it should be mentioned that regulation of senescence-associated gene expression overlaps but is not identical with that of sugar-responsive genes. It is well known that sugar depletion enhances expression of senescence-associated genes mediated by the hexokinase-dependent pathway (Dai *et al*., 1999; Noh and Amasino, 1999; Xiao *et al*., 2000). For example, in transgenic tomato plants, overexpression of hexokinase accelerates leaf senescence and reduces photosynthetic activity (Dai *et al*., 1999). A largely overlapping

pattern of gene expression among dark treatment, sugar depletion and age-related senescence is observed (Azumi and Watanabe, 1991; Park *et al*., 1998; Fujiki *et al*., 2001). However, my findings clearly revealed that not all transcripts that were markedly induced in leaves by darkness and sugar deprivation, were also induced during age-related senescence. This is consistent with the idea that senescence is controlled by various environmental cues, not only by the sugar status, but also by other nutrients and physical conditions (Oh *et al*., 1996; Wingler *et al*., 1998; Weaver *et al*., 1998, Lim *et al*., 2003).

MATERIALS AND METHODS

Plant materials and growth conditions

Arabidopsis (ecotype Columbia) were grown on $0.5 \times$ MS liquid medium (Murashige and Skoog basal salts, pH5.8) containing 2 % sucrose (w/v) under a 16h light/8h dark cycle at 21°C. Three weeks after sowing, seedlings were deprived of sugar by transfer to a sucrose-free medium and further culture in the dark for up to 79 h. Seedlings were re-fed sugar by transfer after culture in sucrose-free medium for 72 h to medium supplemented with 2 % sucrose (w/v) for 7 h.

Preparation of cDNA macroarrays

The *Arabidopsis* cDNA macroarrays were supplied by Kazusa DNA Research Institute

(Kisarazu, Chiba). The filters (8 × 12 cm) contained approximately 13,000 polymerase chain reaction-amplified cDNA fragments prepared from four different tissues: above-ground organs, flower buds, roots and green siliques. The amplicon generation and array production were performed as described earlier (Asamizu *et al.*, 2000; Sasaki *et al.,* 2001).

RNA extraction and macroarray hybridization

Total RNAs were isolated from seedlings using the AGPC (acid guanidinium-phenol-chloroform) method (Suzuki *et al.*, 2001). Poly(A)$^+$RNA was purified from 600 μg of total RNA using the PolyATtract mRNA Isolation System (Promega, Madison WI). One microgram of oligo(dT) and 0.3-μg of poly(A)$^+$RNA in a 12 μL solution were denatured at 70°C for 10 min, cooled on ice for 3 min, and subjected to reverse transcription in a total volume of 28 μl (3 μL buffer, 3 μL 25 mM MgCl$_2$, 1.5 μL10 mM each dNTPs, 1 μL 0.1 M DTT, 6 μL [α-^{33}P] dCTP and 1.5 μL reverse transcriptase) at 37°C for 90 min. The resultant ^{33}P-labeled cDNA probes were purified through Quant^{TM} G-50 Micro columns (Amersham Pharmacia Biotech, Buckinghamshire). After prehybridization for 1 h at 65°C in Church-phosphate buffer, pH 7.2 [0.5 M Na$_2$HPO$_4$, 1 mM EDTA and 7 % sodium dodecyl sulphate (SDS)] supplemented with 10 μl of oligo(dA), macro-array filters were subjected to hybridization with the probes in a total volume of 10 ml at 65°C for 16-20 h. The filters were washed once with 2 × saline sodium citrate (SSC) containing 0.1 % SDS and twice with 0.5 × SSC containing 0.1 % SDC at 65°C for 30 min each. Each macro-array filter was successively

used for alternate hybridization with ^{33}P-labeled cDNA probes prepared from treated or untreated plant materials.

Data analysis

Filters were scanned using a BAS 5000 (Fuji, Tokyo), and signal intensity was analyzed by Array Vision (Amersham Pharmacia Biotech, Buckinghamshire, England) with subtraction of the background. Spots showing values less than the background were excluded from further analysis. To improve comparability between different hybridizations, the quantified signal intensity was standardized with respect to the integrated value per filter. Up- and down-regulated sugar responding genes were identified with the criteria of >3 or <0.3 fold the untreated level.

Sub-array production, hybridization and data analysis

To exclude inconstant clones among genes identified by the initial array screening, a sub-array was constructed using 184 genes and 8 different controls with their adjacent duplicate genes giving a cDNA sub-array with a total of 384 elements (Gene-Lab Co. Ltd., Sapporo, Hokkaido). Hybridization was performed according to the protocol used for the cDNA macro-array analysis. Reproducibility of hybridization signals was estimated from the mean value of triplicate experiments.

RNA gel blot analysis

Total RNAs were extracted from total seedlings or detached leaf tissues as described (Suzuki *et al.*, 2001). Ten micrograms of each RNA sample was size-fractionated by 1.2% formaldehyde-agarose gel electrophoresis, transferred onto a nylon membrane, and cross-linked by UV iradiation. The membrane was subjected to hybridization at 42°C for 16 h, washed twice in 0.5 × SSC, 0.1 % SDS at 65°C, and placed in contact with x-ray film as described (Suzuki *et al.*, 2001).

Determination of soluble sugars

Soluble sugars were extracted with ethanol from leaf samples. A 2-g of leaves was ground in a chilled mortar in 2 mL 80% (v/v) ethanol. Homogenates were transferred to centrifuge tubes and subjected to centrifugation at 8,400 × g for 10 min. The pellets were successively incubated with 2 mL 80 % (v/v) ethanol, and 1 mL 50 % ethanol. Three phases were pooled and incubated at 80°C for 30min. The solution was centrifuged at 8,400 × g for 10min, and supernatant was dried in a Speed Vac Concentrator (Savant Instruments Inc., Holbrook, NY). The dry residue was resuspended in 400 µl of 80 % ethanol, mixed with 400 µl chloroform, and centrifuged at 5,000 × g for 10 min. The supernatant was analyzed for soluble sugars by the enzymatic method (Sigma Diagnostics Glucose reagents, ST. Louis MO). Briefly, the sample was mixed with glucose oxidase, peroxidase and o-dianisidine, and incubated at 37°C for 30 minutes. Sucrose was quantified after complete digestion with invertase.
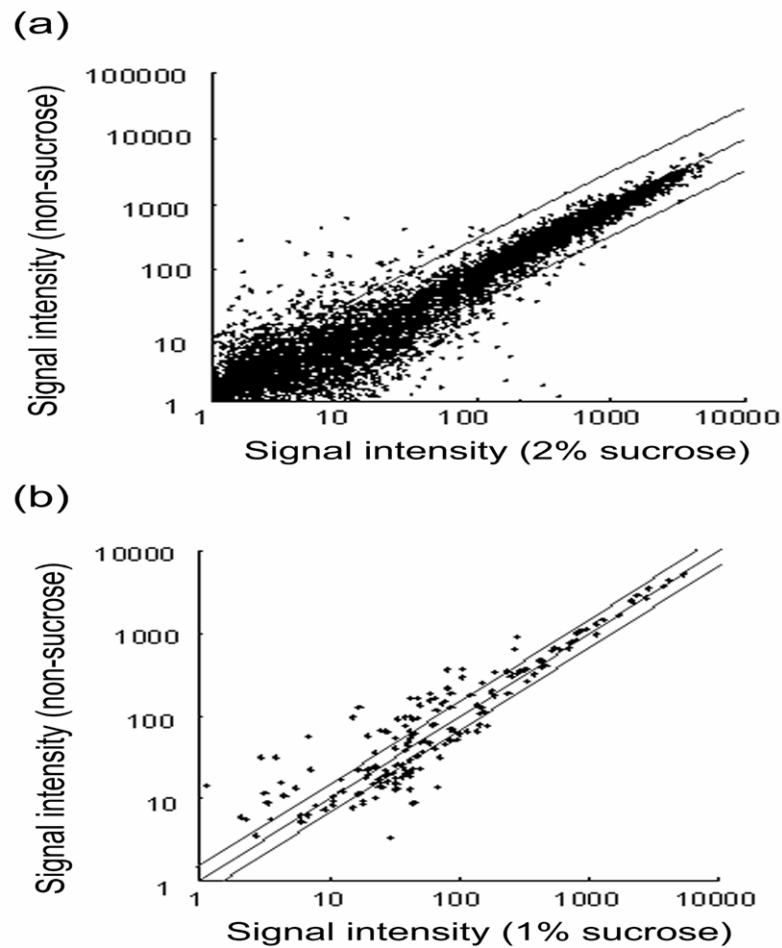
Figure 1-1. Identification of sugar-responsive genes. (a) Initial macro-array assay. For sugar depletion, 3-week-old seedlings grown on growth medium with 16h light/8h dark cycles were deprived of sucrose for 79 h. As the control, seedlings were cultured in the presence of sucrose for 7 h after 72 h sucrose depletion. Total RNAs were isolated and used as the probes. The middle line indicates equivalent signal intensity, and the outer lines indicate the range of 3-fold differences. (b) Sub-array assay. Genes initially selected by the primary screening showing over 3-fold intensity were subjected to sub-array screening. The middle line indicates equivalent signal intensity, and the outer lines indicate the range of 1.5-fold differences. Scores greater than ±1.5-fold were deemed to be significant. All signal intensities are shown as logarithmic values.
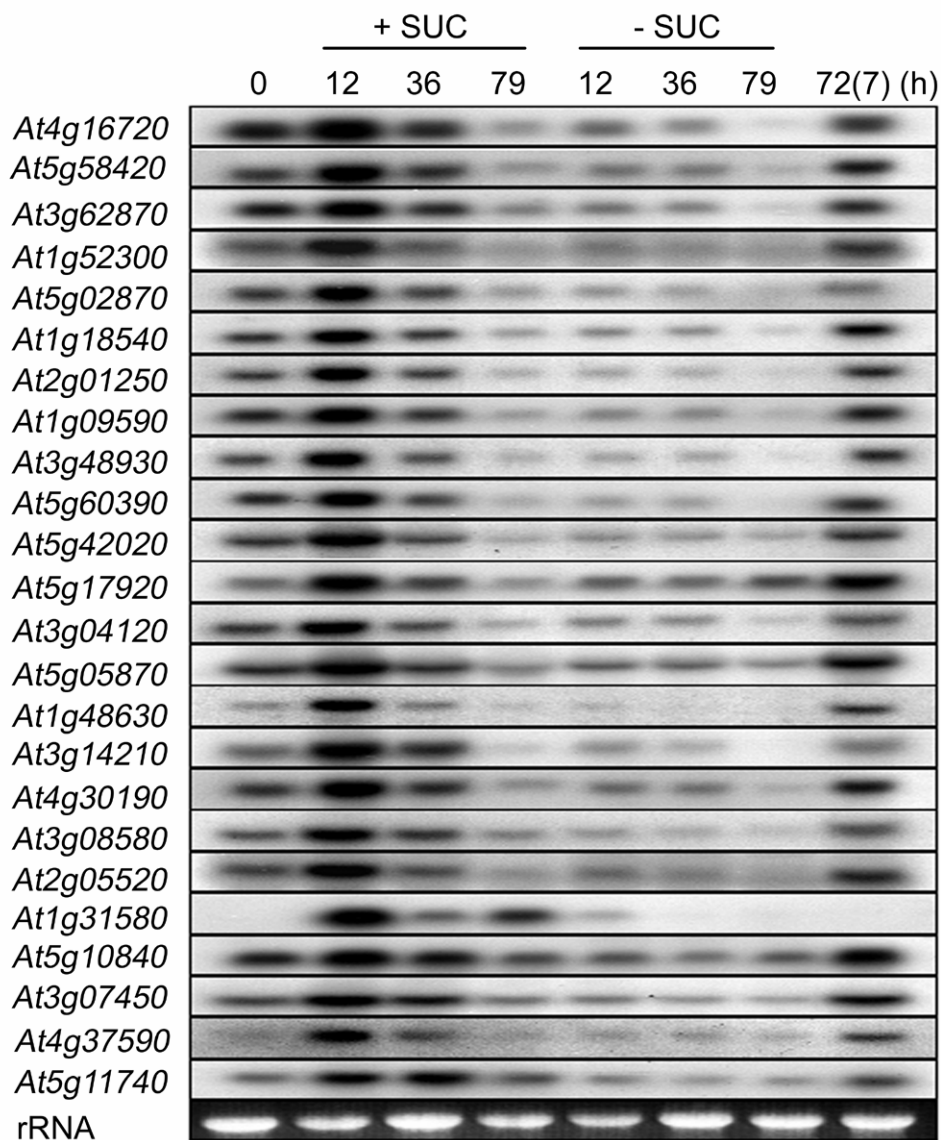
Figure 1-2. RNA gel-blot analysis. Total RNA was extracted from leaves cultured in the presence or absence of sucrose for the indicated time periods. Total RNA was also extracted from leaves supplied with sucrose for 7 h after 72 h sucrose depletion (indicated as 72(7)). A 5-μg aloquot per lane was fractionated on agarose gel electrophoresis, blotted to nylon-membrane and subjected to hybridization with indicated probes. rRNA was visualized with ethidium bromide staining for the loading control.

Table 1-1. Functional classification of down-regulated genes by sugar depletion

Gene ID	Array fold	Description	Predicted function
At4g16720	0.43	Ribosomal protein	Translation machinery
At5g58420	0.5	Ribosomal protein	
At3g62870	0.47	Putative 60S ribosomal protein	
At1g52300	0.58	Putative 60S ribosomal protein	
At5g02870	0.45	Putative 60S ribosomal protein	
At1g18540	0.5	Putative 60S ribosomal protein	
At2g01250	0.52	Putative 60S ribosomal protein	
At1g09590	0.5	Putative 60S ribosomal protein	
At3g48930	0.53	Cytosolic ribosomal protein	
At5g60390	0.61	Translation elongation factor eEF-1 alpha	
At5g42020	0.45	Luminal binding protein (Bip)	
At5g17920	0.5	5-methyletetrahydropteroyltriglutamate -homocysteine S-methyltrasferase	Amino acid biosynthesis
At3g04120	0.7	Glyceraldehydes-3-phosphate dehydrogenase	Organic acid synthesis
At5g05870	0.46	Glucuronosyl transferase-like protein	
At1g48360	0.55	Guanine nucleotide binding protein, putative	
At3g14210	0.46	Myrosinase-associated protein	Secondary metabolites metabolism
At4g30190	0.46	H^+-transporting ATPase Type2	Energy metabolism
At3g08580	0.39	Adenylate translocator	
At2g05520	0.56	Putative glycin-rich protein	Unknown
At1g31580	0.51	ORF1, putative	
At5g10840	0.5	Putative protein	
At3g07450	0.62	Putative 5B-anther specific protein	
At4g37590	0.3	Putative protein	
At5g11740	0.44	Putaitve protein	

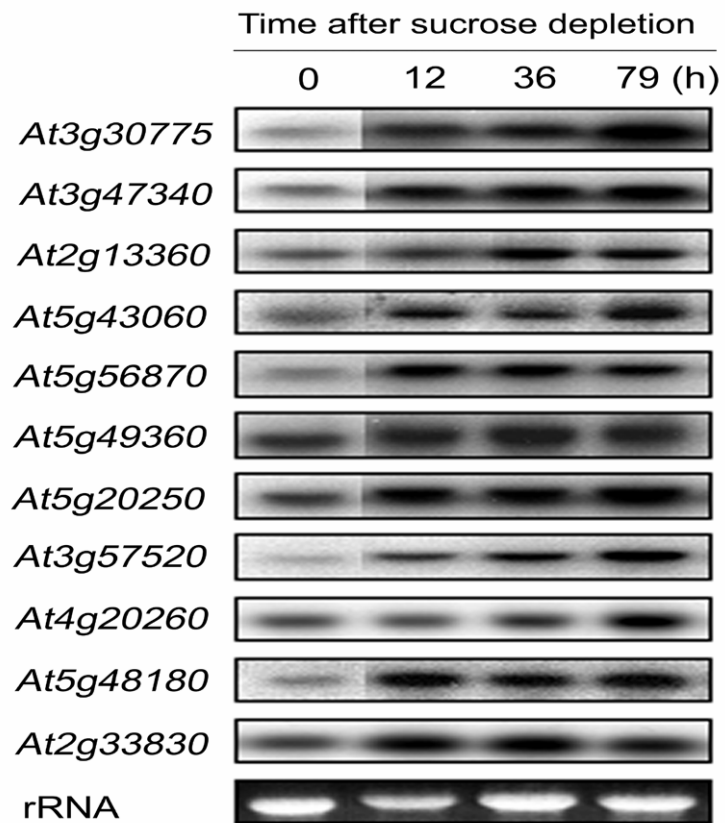**Figure 1-3. Time course of transcript accumulation.** Seedlings were grown on 1/2 MS medium containing 1 % sucrose for 3 weeks under a photoperiod of 16h light/8h dark, and then transferred to 1/2 MS medium without sucrose and further cultured in the darkness for indicated time periods. Transcript levels were analyzed by RNA blot hybridization using a 10-µg aliquot of RNA per lane as described in the legend for Fig. 1-2.

Table 1-2. Functional classification of up-regulated genes by sugar depletion

Gene ID	Array fold	Description	Predicted function
At3g30775	1.72	Proline oxidase	
At3g47340	4.44	Gultamine-dependent asparagine synthetase	
At2g13360	6.24	Alanine glyoxylate aminotransferase	Amino acid metabolism
At5g43060	2.33	Cysteine protease	
At5g56870	2.27	β-galactosidase	
At5g49360	7.7	β-xylosidase	
At5g20250	3.55	Seed imbibition protein	Carbohydrate metabolism
At3g57520	3.70	Imbibition protein homolog	
At4g20260	2.49	Endomembrane associated protein	
At5g48180	10.32	Myrosinase-binding protein-like protein	Unknown
At2g33830	2.07	Putative auxin-regulated protein	

Figure 1-4. Changes in sugar contents and transcript levels during sugar depletion. (a) Sugar contents. Plants were grown under a 16 h light/8 h dark cycle for 25 days. Leaves were then detached and sucrose (upper panel) and glucose (lower panel) contents were estimated during the treatments. Sugar treatments were performed by incubation under darkness in the presence (closed square) or absence (open square) of sucrose for 79 h. Similarly, detached leaves were incubated under darkness in the absence of sucrose for 72 h, then supplied with 1% sucrose, and further incubated for 7 h (closed triangle). (b) RNA blot hybridization. Experiments were performed as described in the legend for Fig. 1-2.
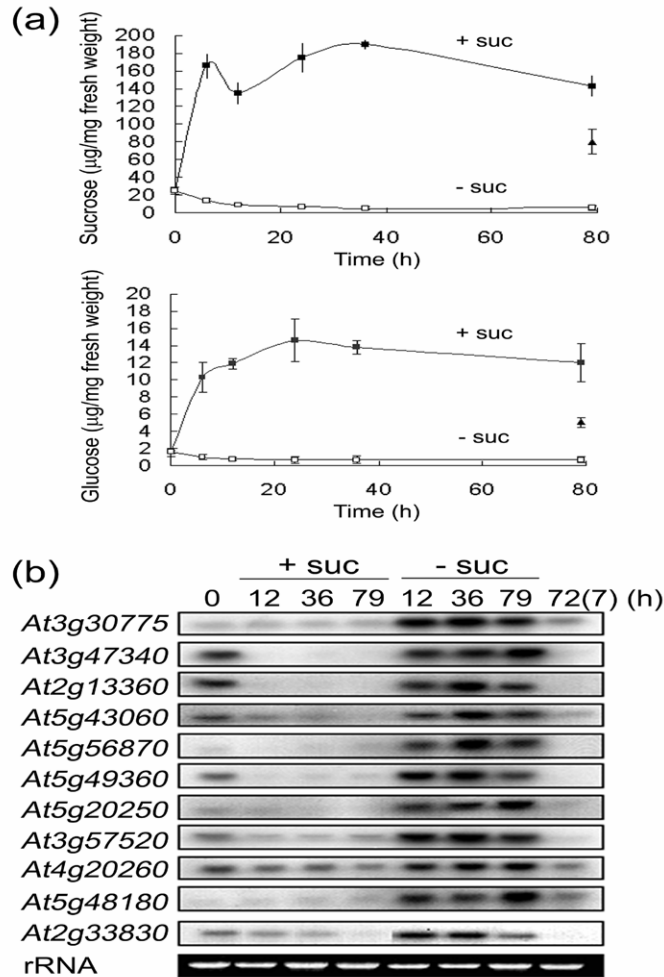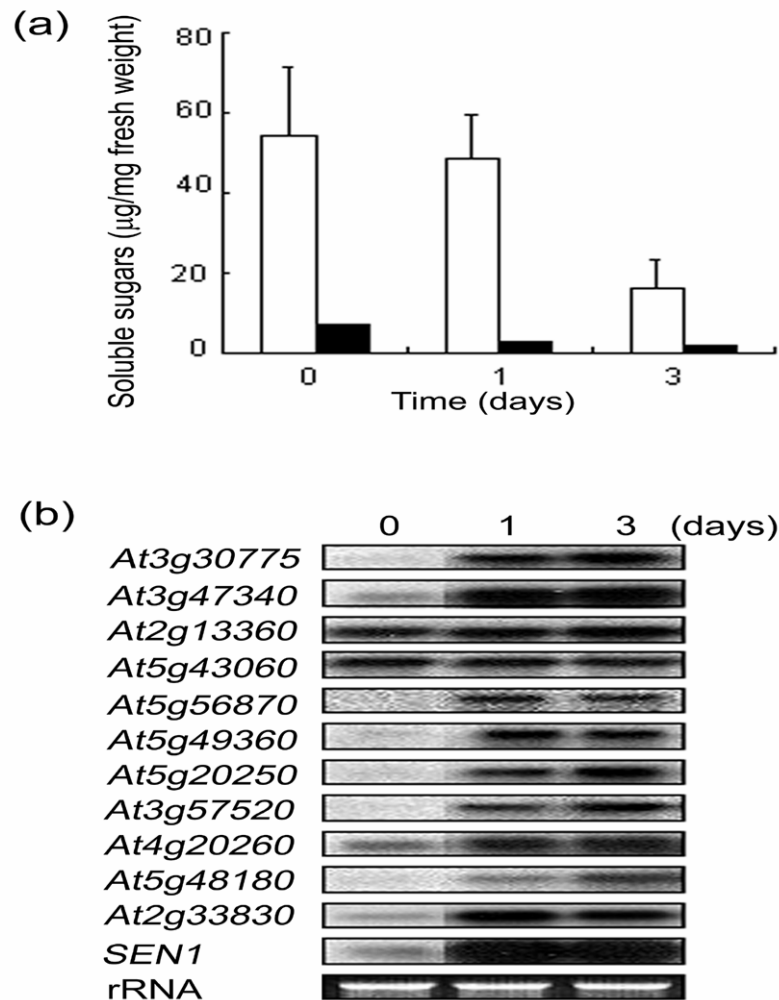
Figure 1-5. Changes in sugar contents and transcript levels in the dark. (a) Sugar contents. Plants were grown under a 16 h light/8 h dark cycle for 25 days after germination, and then transferred to continuous dark conditions. Leaves were detached at the indicated time points and assayed for sucrose (open bar) and glucose (closed bar) contents. (b) RNA blot hybridization. Total RNAs were extracted from leaves detached at the same time as those taken for analysis sugar contents and 5-μg aliquots were assayed for transcript accumulation of the indicated genes by RNA blot hybridization. As a positive control, *SEN1* was used (Shimada *et al*. 1998). Time 0 indicates sampling just before transfer to the dark.
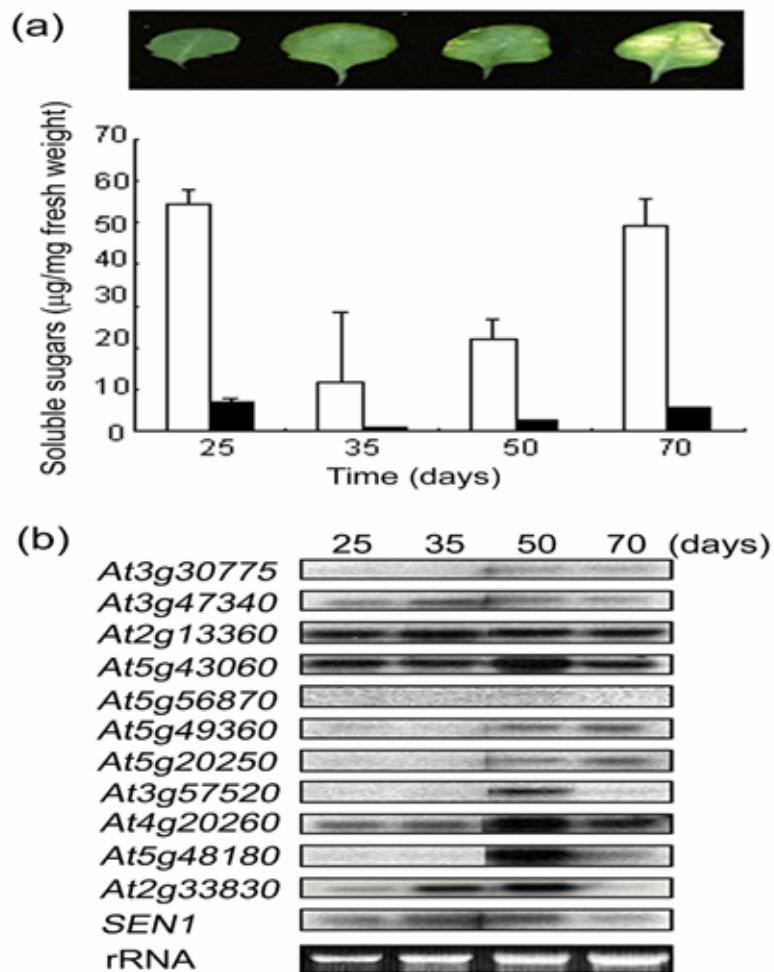
Figure 1-6. Changes in sugar contents and transcript levels during senescence. (a) Phenotype and sugar contents. Plants were grown under a 16 h light/8 h dark cycle after germination, and then leaves were detached at the indicated time points (upper panel) and assayed for sucrose (open bar) and glucose (closed bar) contents (lower panel). (b) RNA blot hybridization. Total RNAs were extracted from detached leaves and 5-μg aliquots were assayed for transcript accumulation of the indicated genes by RNA blot hybridization.

CHARACTERIZATION OF CELL WALL-DEGRADING ENZYMES

Plant cell wall is a fundamental determinant of cell shape, and represents a major carbon sink in the biosphere (Seifert *et al*., 2002). During growth and development, or in response to changing environmental conditions, cell wall likely alters in morphogenesis to require modification (Roulin *et al*., 2002). It is conceivable that, in response to environmental cues, availability of enzymes involved in cell wall-modifications is regulated. Among theses enzymes, those for cell wall degradation have been described, but their physiological role remained to be determined.

In Chapter 1, I identified 11 genes, whose transcripts were induced by sugar starvation. Two genes encoded putative glycoside hydrolases, which were predicted to function in degradation of cell wall components. In this Chapter, I characterized these hydrolases and investigated their roles in cell wall degradation.

RESULTS

Genes encoding glycosyl hydrolase were induced by sugar starvation

Among eleven genes that were induced by sugar starvation in *Arabidopsis* leaves, two genes encoded putative β-galactosidase (At5g56870) and β-xylosidase (At5g49360). BLAST

search of the AGI transcripts database at TAIR revealed that each belonged to a family consisted of fourteen and seven genes, respectively. The phylogenetic trees of nucleotide sequence (Fig. 2-1a, 2-2a) showed considerable homology (55-80 %) among them. In order to ensure whether transcripts of identified genes themselves were prominently induced by sugar starvation, RNA gel-blot analysis was performed. The result showed that transcripts for At5g56870 and At5g49360 were induced under sugar starvation (Fig. 2-1b, 2-2b). In addition to β-galactosidase and β-xylosidase, the gene for β-glucosidase (*At3g60140*), known as the dark-inducible gene 2 (*din2*) (Fujiki et al., 2000), was also transcriptionally activated by sugar starvation (Fig. 2-3). I refer β-galactosidase, β-xylosidase and β-glucosidase as *Gal, Xyl* and *Glu,* respectively, thereafter.

Identified genes encode cell wall-degrading enzymes

Proteins derived from *Gal, Xyl* and *Glu* consisted of 724, 774, and 577 amino acids, respectively, and their molecular masses were calculated as 80.5kDa, 83.5kDa, and 66.9kDa. Corresponding proteins designated as GAL, XYL and GLU had the characteristic regions for glycosyl hydrolases family 35, 3 and 1, respectively. A prediction program for protein localization, PSORT, indicated them to have signal peptides for secretion and several potential *N*-glycosylation sites. Amino acid sequence and schematic structure of these proteins are shown in Fig. 2-4.

Database search indicated that they are similar to cell wall-degrading enzymes. GAL was very similar in sequence with a β-galactosidase of Japanese pear that was reportedly localized in cell wall of fruits and is induced associating with ripening (Tateishi *et al.*, 2001). It was predicted to function in degradation of cell wall during fruit softening. XYL showed a considerable similarity with barley β-glucan exohydrolases detected in germinated barley grain (Hrmova *et al.*, 1996). This hydrolase was one of the candidates for the release of glucose from oligosaccharides derived from (1->3, 1->4)-β-glucans in crude extracts of germinating barley grain (Simos *et al.*, 1994; Leah *et al.*, 1995).

Cell wall-degrading enzymes were characterized using cultured cells

GAL, XYL and GLU appeared to be apoplastic proteins. For confirmation, I used cultured cells, since apoplastic proteins could be easily recovered from growth medium. First, I examined whether transcripts of those genes were also induced in cultured cells by sugar starvation. As shown in Fig. 2-5, their transcripts were induced by sugar starvation and repressed by sucrose feeding. Transcripts were detected within 12 h of sugar starvation, while 10 mM of sucrose almost completely repressed their expression.

Subsequently, activities of β-galactosidase, β-xylosidase and β-glucosidase in culture medium were measured using synthetic substrates. As shown in Fig. 2-6, the level of these enzyme activities elevated when sucrose was removed from the medium. The increase in

β-galactosidase activity began 12 h after onset of sugar starvation, maintaining the high level thereafter during sugar starvation. Activities of β-xylosidase and β-glucosidase in the presence of sucrose markedly differed from those in the absence of sucrose (Fig. 2-6b,c). These results suggested that products of identified genes were secreted into apoplast or culture medium in association with sugar starvation. However, whether or not these activities were due to GAL, XYL and GLU is not clear, since multiple enzymes utilize the synthetic substrates used in this assay.

In order to address this question, anti-GAL antibody was prepared using recombinant GAL protein produced in *Escherichia coli* as the antigen. Immuno-blot analysis showed an accumulation of GAL protein in sugar-deprivated culture medium, but not in the cell extracts (Fig. 2-7).

Cultured cells utilize various monosaccharides as carbon source

GAL, XYL and GLU were considered to be cell wall-degrading enzymes functioning in apoplasts. This led the idea that they release monosaccharides, galactose, xylose and glucose from cell wall materials which are utilized as carbon source in cell. In order to examine this idea, cultured cells were grown in the presence of one of such sugars. Results showed that cultured cells grew in the medium containing glucose, galactose, fructose, or xylose, while they did not grow without sugar supply (Fig. 2-8). This result indicated that plant cells could

utilize their monosaccaride. Furthermore, glucose, galactose, fructose, and xylose were as effective as sucrose for repressing expression of these genes (Fig. 2-9).

Cell wall polysaccharides decreased during sugar starvation

Composition of cell wall materials in leaves was directly analyzed. Detached leaves were floated on water with or without 1 % sucrose in darkness up to three days. Cell wall components were fractionated according to the sequential treatments of EDTA, KOH, and H_2O_4S. Total sugar residues of each fraction was measured with the phenol-sulfuric acid method and normalized to glucose equivalents. As shown in Table 2-1, amounts of pectin, hemicellulose I and hemicellulose II decreased during sugar starvation, while they were rather constant when supplied with sucrose. In particular, levels of pectin and hemicellulose I decreased approximately to two third of the control after 3 days of sugar starvation. In contrast the amount of cellulose did not significantly change.

Transcripts of Gal, Xyl and Glu were induced in various organs by inhibition of photosynthesis

Transcripts of *Gal*, *Xyl* and *Glu* were induced in detached leaves and cultured cells under sugar starved condition. To examine whether this is also the case *in planta*, various organs including mature rosette leaves, basal part of the stem, flowers, siliques, and cauline leaves

were assayed after plants were kept in darkness for 24 h to induce natural sugar starvation. RNA gel-blot analysis showed their transcripts were weakly accumulated when plants were grown under normal condition, and significantly increased in all organs when subjected to sugar starvation (Fig. 2-10).

Subsequently, pattern of *Gal* expression was investigated for tissue specificity using transgenic plants harboring a chimeirc gene consisted of the *Gal* promoter and β-glucuronidase (GUS) gene. GUS activity was relatively low when plants were grown under normal condition (Fig. 2-11a). When organs were kept under sugar-starved condition, GUS activity increased in flowers, rosette leaves, and rays (Fig. 2-11b). This result indicated that GUS activity reflected the expression profile of endogenous *Gal* gene. GUS activity was observed in vein of cotyledons and emerging true leaves in 10-day-old seedlings (Fig. 2-11c). When plants were placed in the darkness for 1 and 2 days, strong GUS staining was observed in rosette leaves and flowers being consistent with the results of RNA gel-blot analysis (Fig. 2-10 and 2-11d,e). When a part of rosette leaves was covered with aluminium foil to repress photosynthesis, covered region was strongly stained (Fig. 2-11f).

How is expression of Gal, Xyl and Glu regulated?

Transcripts for *Gal*, *Xyl* and *Glu* genes encoding putative enzymes for cell wall degradation were induced by sugar starvation. This raised further questions how these genes were

regulated by sugars. Hexokinase is often required for glucose signaling, which differs for its enzymatic activity. This signaling feature was examined by glucose analogs, which are widely used to investigate the relation of hexokinase in sugar signaling pathway. Results showed that neither 2-deoxy-glucose nor 3-*O*-methyl-glucose repressed expression of *Gal*, *Xyl* and *Glu*, indicating hexokinase was not involved in their regulation (Fig. 2-8). Since several hexoses similarly repressed their transcript accumulation (see Fig. 2-9), it was speculated that metabolites of hexose functioned as signaling molecule rather sucrose itself.

DISCUSSION

Sugar starvation induced transcripts of three genes encoding putative glycosyl hydrolases, *Gal*, *Xyl* and *Glu*. Their deduced polypeptides indicated the presence of signal peptides for secretion. GAL showed sequence similarity with the β-galactosidase of Japanese pear, which was induced during fruit softening. This enzyme was proposed to play a role in degradation of cell wall components. The β-galactosidase, forms a family of glycosyl hydrolases, showing substrate specificities, and localizes to diverse subcellular and extracellular locations (Dey and del Campillo, 1984; Singh and Knox, 1984; Kulilova *et al*., 1990). Some were thought to play a role in cell wall degradation. For instance, a *Nasturtium* β-ᴅ-galactosidase was found to be able to remove the terminal β-ᴅ-galactosyl residue from xyloglucan side-chains (Edwards

et al., 1988). Indeed, β-galactosidase is the only enzyme that has been shown to cleave the β-(1->4)-galactan bond directly and produce galactosyl residues from the cell wall during ripening (Smith *et al*., 1998).

β-xylosidases are currently classified into five families of glycosyl hydrolases. In fungi, β-xylosidase activity was reported to be rate-limiting in xylan hydrolysis, liberating xylose from non-reducing termini of soluble xylose oligomers during protection pathogen attack (Van Peij *et al*., 1997). During the course of my study, *AtBXL1*, a gene encoding a putative β-xylosidase was identified (Goujon *et al*., 2003). The sequence analysis indicated it to be identical my *Xyl*. Antisense suppression of *AtBXL1* resulted in decrease of β-xylosidase activity and abnormality of leaf shape (Goujon *et al*., 2003). The authors suggested that *AtBXL1* was involved in hemicellulose metabolism, but did not indicate the possibility that its functions in sugar supply (Goujon *et al*., 2003). Roulin et al. reported that activities of (1->3, 1->4)-β-D-glucan endohydrolase isoenzyme I and β-D-glucan glucohydrolase increased in the leaves of barley seedlings when they were incubated in the dark. 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. Indeed, β-glucosidases were proposed to have many functions in cellular processes during normal growth and development of plants, depending on their substrate specificities (Leah *et al*., 1995).

Based on the present results together with above-mentioned information, I propose that GAL, XYL and GLU release galactose, xylose and glucose, respectively, from the cell wall. These monosaccharides could be transported into the cytoplasm from the apoplast and then used as the carbon source to cope with sugar starvation. Subsequent experiments partly confirmed this hypothesis, showing higher activities of β-galactosidase, β-xylosidase and β-glucosidase were observed in sugar-depleted medium. Immunological assay also showed GAL protein in culture medium depleted with sucrose. Direct measuring contents of cell wall material indicated that pectin and hemicellulose I clearly decreased when detached leaves were incubated in dark for three days. It is established that pectin consists of galaturonic acid, rhamnose, arabinose, and galactose (Dey and Brinson, 1984), and that hemicellulose consists of mannose, xylose, arabinose, galactose, and glucose (Dey and Brinson, 1984). It was thus highly conceivable that decrease of both fractions under sugar starvation was due to release of galactose, xylose, and glucose by actions of GAL, XYL and GLU, respectively.

Transcripts for three hydrolases were clearly induced by sugar starvation, or repressed by sugars, suggesting the presence of regulation system by sugar sensing. Its molecular basis has been intensively studied, and hexokinase was repeatedly proposed to play the key role (Jang *et al*., 1997; Halford *et al*., 1999; Jang and Sheen 1994, Moore *et al*., 2003). To diagnose its participation in sugar response, glucose analogs including 3-*O*-methyl-glucose and 2-deoxy-glucose were after used (Graham *et al*., 1994; Klein and Stitt, 1998; Ho *et al*., 2001).

In the present study, I also examined effects of these analogs, and found that transcript induction of three genes was not affected. This suggested that their signal transduction was hexokinase-independent.

Taken together, this chapter provides evidences that one of functions of cell wall is to serve as carbon source being degraded by hydrolases upon sugar starvation.

MATERIALS AND METHODS

Plant material and treatments

The *Arabidopsis* suspension-cultured cells were obtained from Prof. Okada (Kyoto University) and maintained by transplanting a 15 mL of the cell suspension in stationary phase to 35 mL of fresh MS medium (Murashige and Skoog basal salts, pH 5.8) containing 3 %(w/v) sucrose every week. Cultures were grown in flasks at 22   with a rotation. For sugar starvation treatments, 7-day-old growing cells were collected on a filter paper using vacuum filtration, and re-suspended in MS medium without sucrose. As a control, cells were rinsed and incubated with fresh medium containing 3 % (w/v) sucrose. In the case of detached leaves, treatments were performed as described in the Materials and Methods for Chapter I.

Sequence analysis

Nucleotide and deduced amino acid sequence comparisons against the databases were done

using BLAST searches. Signal sequence prediction was conducted using the interactive web sites PSORT (Nakai and Kanehisa, 1992).

RNA gel-blot analysis

Isolation of total RNA from suspension-cultured cells and RNA gel-blot analysis were performed as described previously in Chapter I.

Enzyme assays

After removal of cells by filtration, 25 mL of 0.5 M sodium phosphate (pH 7.4) and 49 g of ammonium sulfate (60 % saturation) were added to 100 mL of the 7 d grown medium, and stirred on ice for 2 h. After centrifugation at 10,000 × g for 20 min, the pellet was re-suspended 1 mL 0.5 M sodium phosphate (pH 7.4) and desalted by NAP-5 column (Amersham Pharmacia Biotech, Buckinghamshire, England). The activities of β-galactosidase, β-xylosidase and β-glucosidase were determined using the corresponding 4-methylumbelliferyl glycopyranoside substrates. The assay was performed with 50 μL protein solution obtained from media and 2 mM substrate in 1 mL of 0.5 M sodium phosphate buffer (pH 5). After incubation for 1 h at 37, the reaction was terminated by the addition of 1 mL of 200 mM sodium carbonate, and fluorescence of 4-methylumbelliferone was quantified from its absorbance at 460 nm.

Production of recombinat GAL and its antibody

The DNA region encoding the putative mature GAL protein was amplified by polymerase chain reaction (PCR), cloned into a pBAD/D-TOPO® vector in frame with HP-thioredoxin and C-terminal peptide containing the V5 epitope and a polyhistidine (6× His) tag. The recombinant vector was transformed into One Shot® TOP10 *Escherichia coli* (Invitrogen, Carlsbad, California). Recombinant GAL protein was purified by MBL Co. (Nagano, Japan). Antibody production was performed by injecting the purified recombinant *GAL* protein into rabbit.

Protein isolation and immunoblot analysis

Total soluble protein was isolated from suspension-cultured cells by homogenization in 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.05 % Tween 20, 1 mM EDTA, 250 μM PMSF, and the extract was clarified by centrifugation at $20,000 \times g$ for 10 min. Proteins extracted from cells and media were separated on a 10 % SDS-polyacrylamide gel electrophoresis. Tris-Gly buffer (25 mM Tris, 192 mM Glycine, 20 % methanol, and 0.1 % SDS) was used to transfer of proteins to polyvinylidene difluoride membrane using the Trans-Blot SD cell system (Bio-RAD, Hercules, California). The immunoblot was blocked with blocking buffer (1× PBST; 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 0.1 % Tween 20, and 45 % skim milk) prior to use, and probed with anti-GAL and anti-PEPC (*At2g42600*; phosphoenolpyruvate carboxylase) antibodies in blocking buffer. After excess antibody was

removed with extensive washing in 1× PBST, the secondary antibody, (goat anti-rabbit IgG(H+L)-HRP conjugate, Bio-RAD, Hercules, California) diluted 1:1000 (v/v) in blocking buffer was applied, and extensively washed as described above. Antigen/antibody complex was visualized using the ECL western blotting detection reagent (Amersham Pharmacia Biotech, Buckinghamshire).

Determination of cell wall polysaccharides contents

Methanol-boiled samples were re-hydrated with water and homogenized. Cell wall polysaccharides were fractioned by the method of Hoson *et al*. (1995). The homogenate was washed sequentially with water, acetone, a methanol:chloroform mixture (1:1, v/v) and ethanol, and treated with 2 units ml^{-1} porcine pancreatic α-amylase (EC 3.2.1.1; type I-A; Sigma, St Louis, Mo.) in 50 mM sodium acetate buffer (pH 6.5) at 37 for 3 h. After amylase treatment, pectin fraction was extracted with 50 mM EDTA at 95 and hemicellulose I and II were successively extracted with 4 % (w/v) KOH and 24 % (w/v) KOH containing 0.02 % NaBH$_4$ at 25 , respectively. 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 , dissolved in 72 % (v/v) sulfuric acid for 1 h at 25 , and then diluted with a 29-fold volume of water. Total sugar content in each fraction was determined by the phenol-sulfuric acid method (Dubois *et al*., 1956) and expressed as glucose equivalents.

GUS staining

Seedlings and excised organs, which were subjected to sugar starvation and dark treatment, were placed directly in buffer containing 1.5 mM $K_3Fe(CN)_6$, 1.5 mM $K_4Fe(CN)_6$, 0.9 % Triton X-100, and 5.7 mM X-gluc. The 38.3 mM X-gluc stock in *N,N*-dimethylformaldehyde was freshly prepared every time before use. The samples were vaccum infiltrated for 10 min and incubated at 37   for overnight. After successive clearing in 20 %, 50 %, and 80 % for 5 min, samples were incubated in 99 % ethanol overnight and photographed through a dissecting microscope.
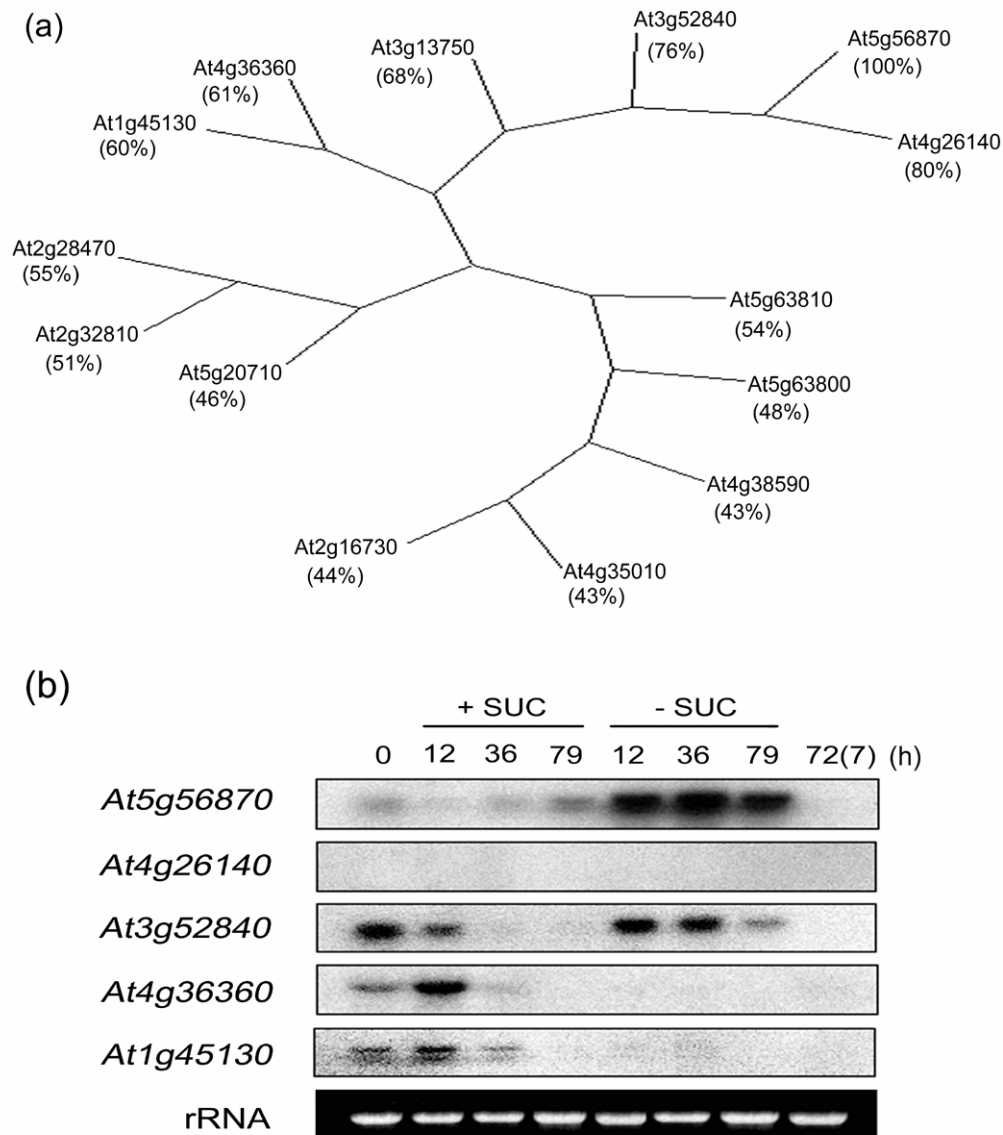
Figure 2-1. Properties of β-galactosidase and related genes. (a) A phylogenetic tree of β-galactosidase and related genes in *Arabidopsis*. The number in a parenthesis indicates the identity with β-galactosidase. (b) Effect of sucrose on transcript accumulation. Sugar treatments were performed by incubation under darkness in the presence or absence of sucrose for 79 h. Similarly, detached leaves were incubated under darkness in the absence of sucrose for 72 h, then supplied with 1% sucrose, and further incubated for 7 h. Total RNAs were extracted from detached leaves during the treatments and hybridized with the probe indicated.
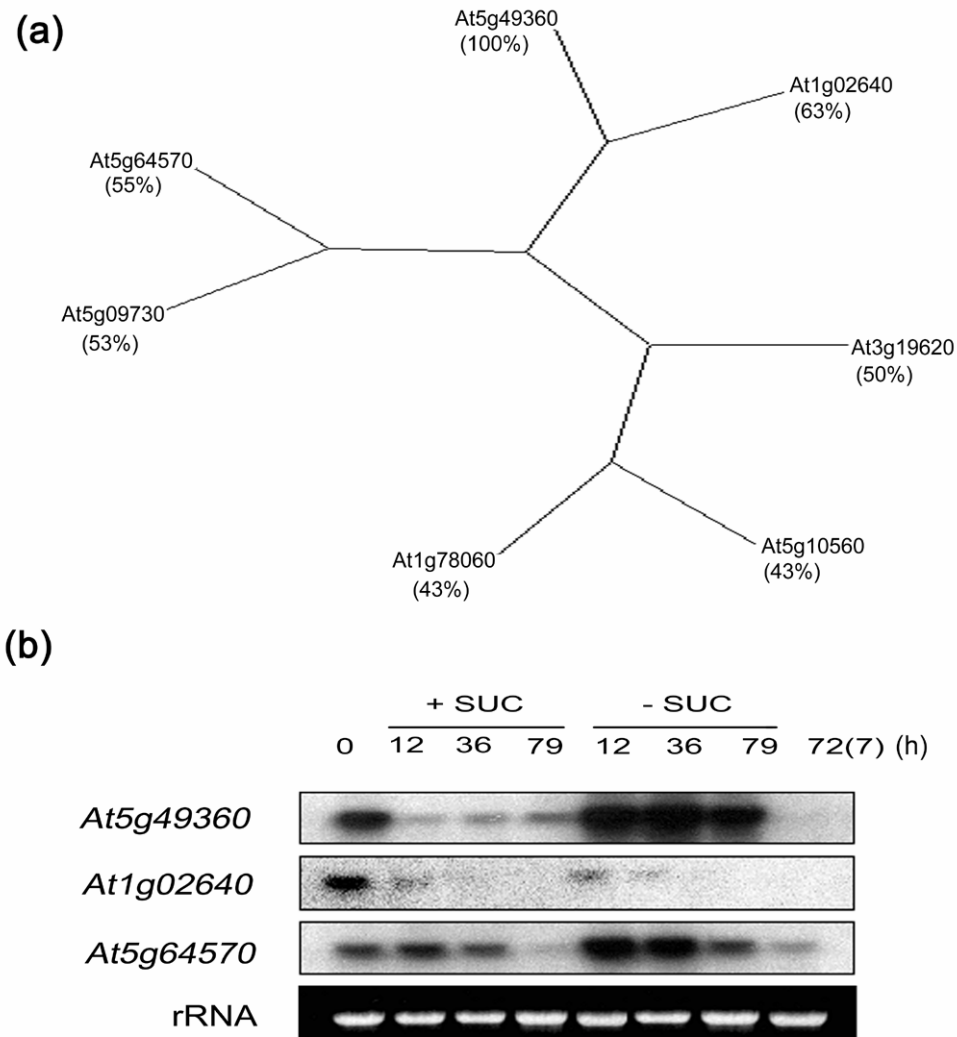
Figure 2-2. Properties of b-xylosidase and related genes. (a) A phylogenetic tree of β-xylosidase and related genes in *Arabidopsis*. The number in a parenthesis indicates the identity with β-xylosidase. (b) Effect of sucrose on transcript accumulation. Sugar treatments were performed by incubation under darkness in the presence or absence of sucrose for 79 h. Similarly, detached leaves were incubated under darkness in the absence of sucrose for 72 h, then supplied with 1% sucrose, and further incubated for 7 h. Total RNAs were extracted from detached leaves during the treatments and hybridized with the probe indicated.
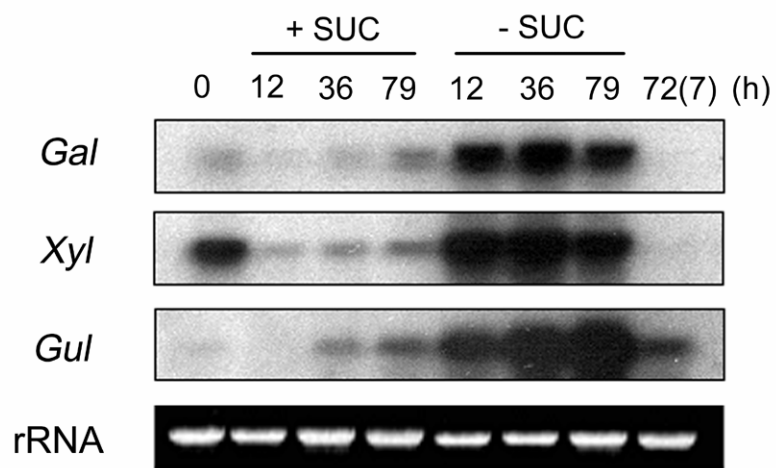
Figure 2-3. RNA gel-blot analysis. Detached leaves were treated in 1% (w/v) sucrose-containing water for 79 h, sucrose-free water for 79 h, or sucrose-free water for 72 h and transferred to sucrose-containing water for an additional 7 h. Total RNA was prepared from each sample and a 5-μg aloquot per lane, were subjected to hybridization with indicated probe.

(a)

MVLNFPRDKSC IPLAILCCLS LSCIVKASVS YDRKAVIING QRRILLSGSI 50

HYPRSTPEMW PGLIQKAKEG GLDVIETYVF WNGHEPSPGQ YYFGDRYDLV 100

KFIKLVHQAG LIYMLRIGPY VCAEWNFGGF PVWLKFVPGM APRTDMEPPK 150

AAMKKFTEKI VWMMKAEKLF QTQGGPIILA QIENEYGPVE WEIGAPGKAY 200

TKWVAQMALG LSTGVPWIMC KQEDAPGPII DTCNGYYCED FKPNSINKPK 250

MWTENWTGWY TDFGGAVPTR PVEDIAYSVA RFIQKGGSLV NYIMYHGGTN 300

FDRTAGEFMA SSYDYDAPLD EYGLPREPKY SHLKALHKAI KLSEPALLSA 350

DATVTSLGAK QEATVPWSKS SCAAFLSNKD ENSAARVLFR GFPYDLPPWS 400

VSILPDCKTE VYNTAKVMAP SVHRNMVPTG TKPSWGSPNE ATPTANEAGT 450

PARNGLVEQI SMTWDKSDTF WIITDITIGS GETFLKTGDS PLLTVMSAGH 500

ALHVFVNGQL SGTAYGGLDH PKLTFSQKIA LHAGVNKIAL LSVAVGLPNV 550

GTHFEQNWKG VLGPVTLKGV NSGTWDMSKW KWSYIKIGVKG EALSLHTNTE 600

SSGVRWTQGS FVAKKQPLTW YKSTFATPAG NEPLALDMNT MGKGQVWIING 650

RNIGRHWPAY KAQGSCGRCN YAGTPDAKKC LSNCGEASQR WYHVPRSWLK 700

SQMLIVVFEE LGGDPNGISL VKRT* 724

(b)

MSCYNKALLI GNKVVVILVF LLCLVHSSES LRPLFACDPA NGLTRTLRFC 50

RANVPIHVRV QDLLGRLTLQ EKIRNLVNNA AAVPRLGIGG YEMWSEALHG 100

ISDVGPGAKF GGAFPGATSF PQVITTAASF MQSLWEEIGR VVSDEARAMY 150

NGGVAGLTTW SPNVNILRDP RWGRGQETPG EDPIVAAKYA ASTVRGLQGT 200

AAGNRLKVAA CCKHYTAIDL DNWWNGVDRFH FNAKVTQQDL EDTYNVPPKS 250

CVYEGKVASV MCSYNQVNGK PTCADENLLK NTIRGQWRLN GYIVSDCDSV 300

DVFPNQQHYT STPEEAAARS IKAGLDLDCG PFLAIFTEGA VKKGLLTEND 350

INLALANTLT VQMRLGMFDG NLGPYANLGP RDVCTPAHKH LALEAAHQGI 400

VLLKNSARSL PLSPRRHRTV AVIGPNSDVT ETMIGNYAGK ACAYTSPLQG 450

ISRYARTLHQ AGCAGVACKG NQGFGAAEAA AREADATVLV MGLDQSIPAE 500

TRDRTGLLLP GYQQDLVTRV AQASRGPVIL VLMSGGPIDV TPAKNDPRVA 550

AIIWAGTPGQ AGGAAIANII PGAANPGGKL PMTWTPQDTV AKVPMTVMAM 600

RASGHYPGRT YRFYKGPVVF PFGFGLSYTT FTHSLAKSPL AQLSVSLSNL 650

NSANTILNSS SHSIKVSHTN CNSPPKMPLH VEVSNTGEPD GTHTVFVPAE 700

PPINGIKGLG VNKQLIAPEK VHVMAGAKQT VQVDVDACKH LGVVDEYGKR 750

RIPMGEHKLH IGDLKHTILV QPQL* 774

(c)

MAKGSWPFII LPIISMLENW INSLELDRHS PPDDPIPGTA ASAPQYEGAT 50

SEGGKSPTIW DHFSLTTPER TKMHNADVAI DFYHRYKDDI KLMKELNMDA 100

PRPSISWSRL IPSGKLKDGV NKEGVQFYKD LIDELLANDI QPSMTLYHWD 150

HPQSLEDEYG GFLSPKIVED FRDFARICFE EFGDKVKMWT TINEPYIMTV 200

AGTDQGNKAA GRCSKWVNEK CQAGDSSTEP YIVSHHTLLA HAAAVEEFRK 250

CEKTSHDGQI GIVLSPRWFE PYHSDSTDDK EAAERALAFE IGWHLDPVIH 300

GDYPEIVKKY AGNKLPSPTV EQSKMLQNSS DFVGINYTTA RFAAHLPHID 350

PEKPRFKTDH HVEWKLTMHS GHIIGPGEER GFLFSHPEGL RKVLNYIKER 400

YNNMPVYIKE NGINDNDDGT KPREEIVKDT FRIEYHKTHF EELHKAIVED 450

GCDVRGTYAW SLMDNFEWEH GYTARFGLIY VDPVNGLKRY PKDSVKWFKR 500

PLKKSVVGES NKEEVEEMSR AEGNKTFKGF EESAGPFASF MAMNQSRRDE 550

ENNRCSFDFP HTHPGVLQGI ENPSSFY* 577

(d) GAL

XYL

GLU

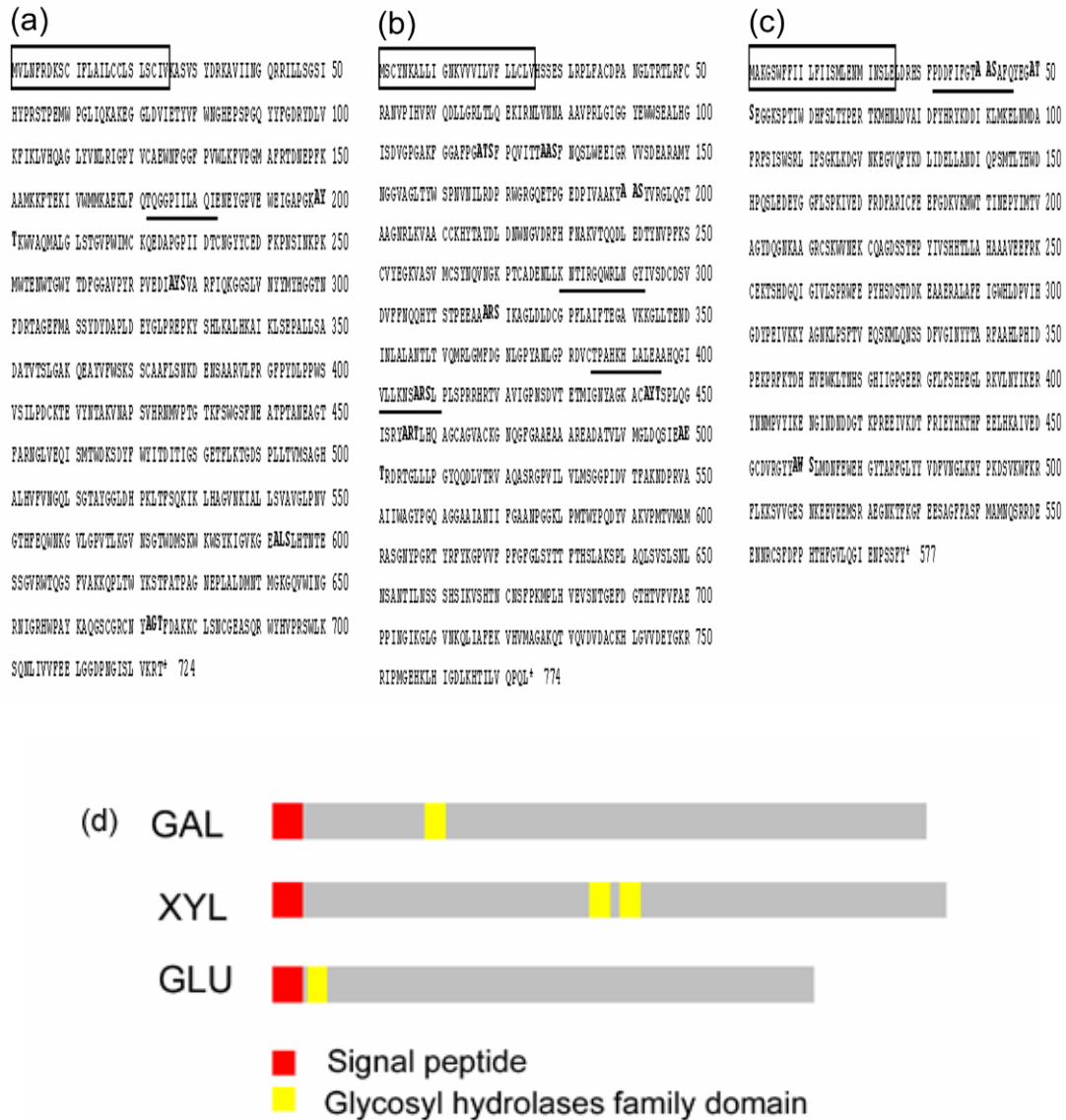■ Signal peptide
■ Glycosyl hydrolases family domain

Figure 2-4. Amino acid sequence and protein structure. (a) Amino acid sequence of GAL. Rectangle: signal peptide; underlined: glycosyl-hydrolase family 35 domain ; bold-letter: putative *N*-glycosylation sites. (b) Amino acid sequence of XYL. Rectangle: signal peptide; underlined: glycosyl-hydrolase family 3 domain; bold-letter: putative *N*-glycosylation sites. (c) Amino acid sequence of GLU. Rectangle: signal peptide; underlined: glycosyl-hydrolase family 1 domain ; bold-letter: putative *N*-glycosylation sites. (d) Structure of predicted proteins. All predicted proteins have a potential signal peptide of 28 amino acids long for apoplastic localization, glycosyl-hydrolase family domain, and several potential *N*-glycosylation sites.
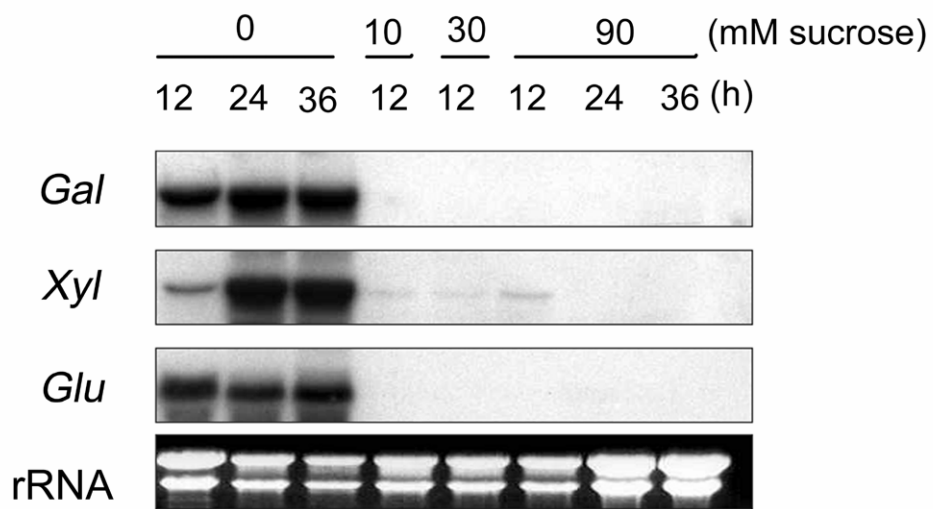
Figure 2-5. Effects of sucrose on transcript accumulation of cell wall-degrading genes. Seven-day-old cells were rinsed and incubated on a medium containing various concentrations of sucrose for 12, 24, or 36 h in darkness. An equal amount of RNA (10-μg) was loaded per lane and analyzed by RNA gel-blot analysis.

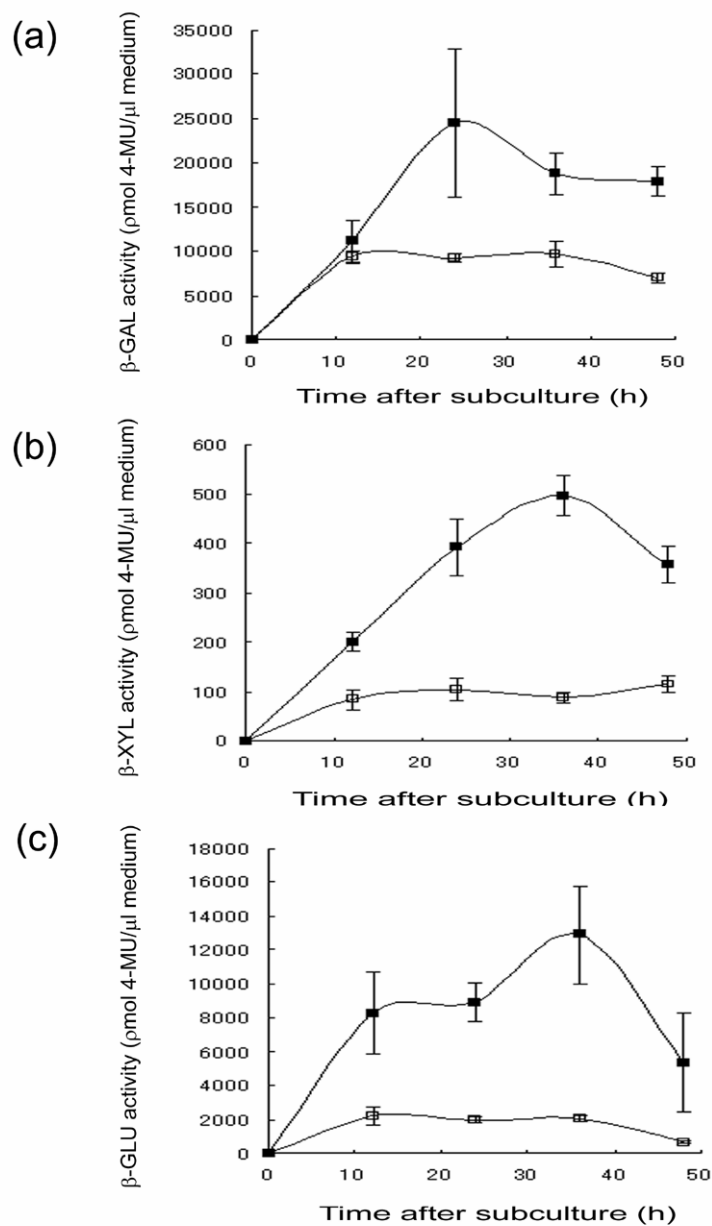**Figure 2-6. Enzymatic activities during sugar starvation.** After cells were grown in the presence or absence of 3 % (w/v) sucrose, the culture medium was harvested every 12 h as the source for enzyme assays. The extracts were assayed for β-galactosidase (a), β-xylosidase (b), and β-glucosidase (c) activity using the enzymatic hydrolysis of 4-methyl-umbelliferon (MU). Open squares indicate sucrose-treatment and closed squares indicate non-treated control samples.
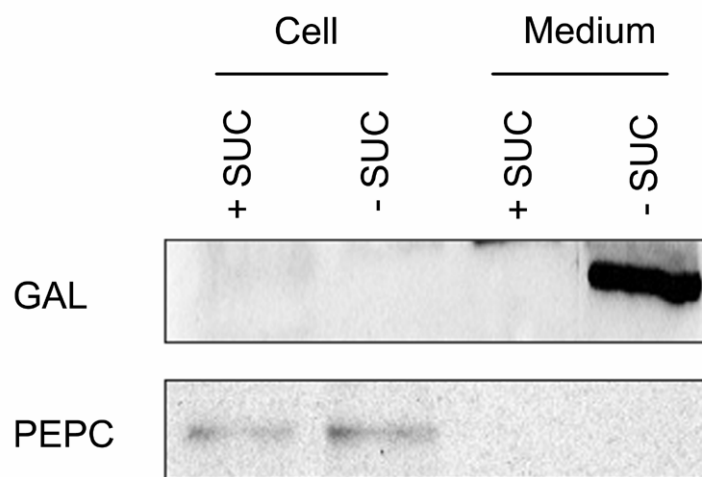
Figure 2-7. Effect of sugar starvation on GAL secretion. Cells and media were collected 48 h after cells transferred to media with or without 3 % (w/v) sucrose, subjected to SDS-PAGE (10 %) and to immunoblot analysis using different antisera against GAL or PEPC. Anti-PEPC antibody was used as the control to recognize intracellular localization.

Table 2-1. Effect of sugar starvation on cell wall polysaccharides

	Polysaccharides (µg/mg fresh weight)							
	Pectin		HC-I		HC-II		Cellulose	
	S+	S-	S+	S-	S+	S-	S+	S-
0 d	4.3±0.8	4.3±0.8	1.3±0.2	1.3±0.2	2.2±0.4	2.2±0.4	3.1	3.1
1 d	4.1±1.5	3.4±0.5	1.3±0.3	1.1±0.1	2.8±1.2	2.1±0.4	3.2±0.7	3.3±1.2
2 d	3.4±0.2	3.0±0.3	1.5±0.1	0.9±0.1	2.4±0.2	1.9±0.3	5.2±0.8	4.6±1.0
3 d	4.2±0.8	2.7±0.8	1.4±0.1	0.9±0.1	3.3±0.8	1.8±0.5	4.4±0.9	3.4±1.6

Different fractions of cell wall components were prepared from leaves treated with the absence (S-) or presence (S+) of 1% (w/v) sucrose for 1-3 days under dark condition. Total sugar contents in each fraction were determined by the phenol-sulfuric acid method and expressed as glucose equivalents. Data represent means of three determinations with standard deviations (±).
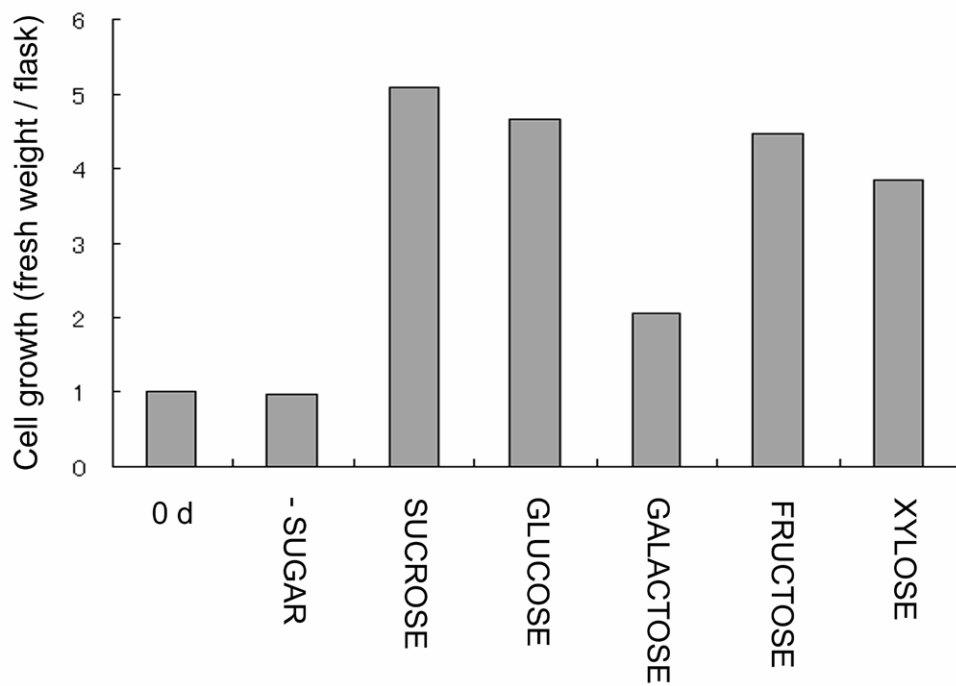
Figure 2-8. Effects of carbon source on cell growth. Cultured cells were incubated in media containing indicated carbon compounds at 90 mM in each treatment, or no sugar supplement. After 7 days incubation, wet fresh weight was estimated.
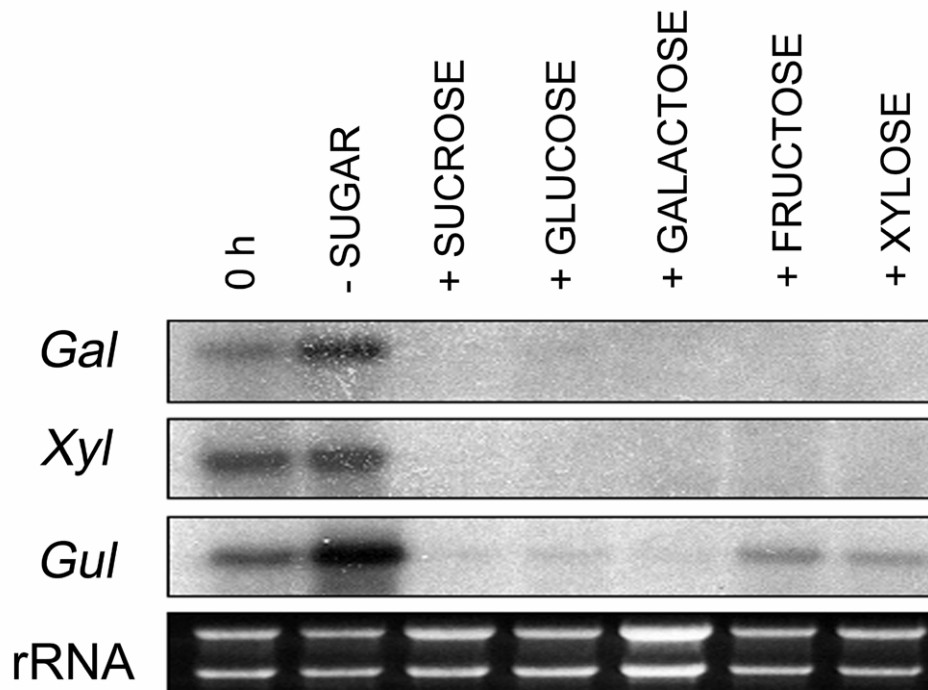
Figure 2-9. Effect of carbon source. Cells were rinsed and transferred into media containing each carbon compound (90 mM), or no sugar for 48 h. A 10-µg of total RNA was loaded per lane and subjected to hybridization with indicated probe.
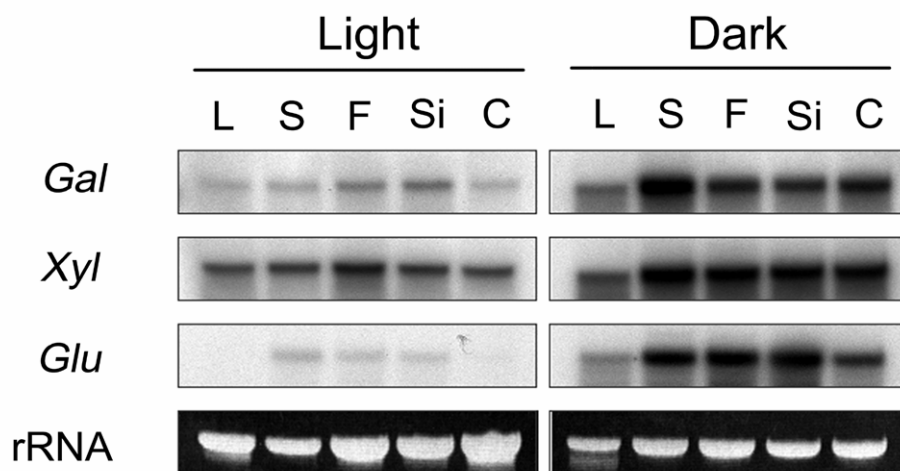
Figure 2-10. Transcript accumulation of cell wall-degrading genes. Total RNA was extracted from rosette leaves (L), stems (s), flowers (F), siliques (Si), and cauline leaves (C) of the 3-week-old *Arabidopsis* plants grown under 16 h light/ 8 h dark cycle. After gel electrophoresis, samples were hybridized with full-length cDNA probe of either *Gal*, *Xyl*, or *Glu*. rRNA was used as the control to ensure equal loading of RNA, and visualized with staining with ethidium bromide.

56
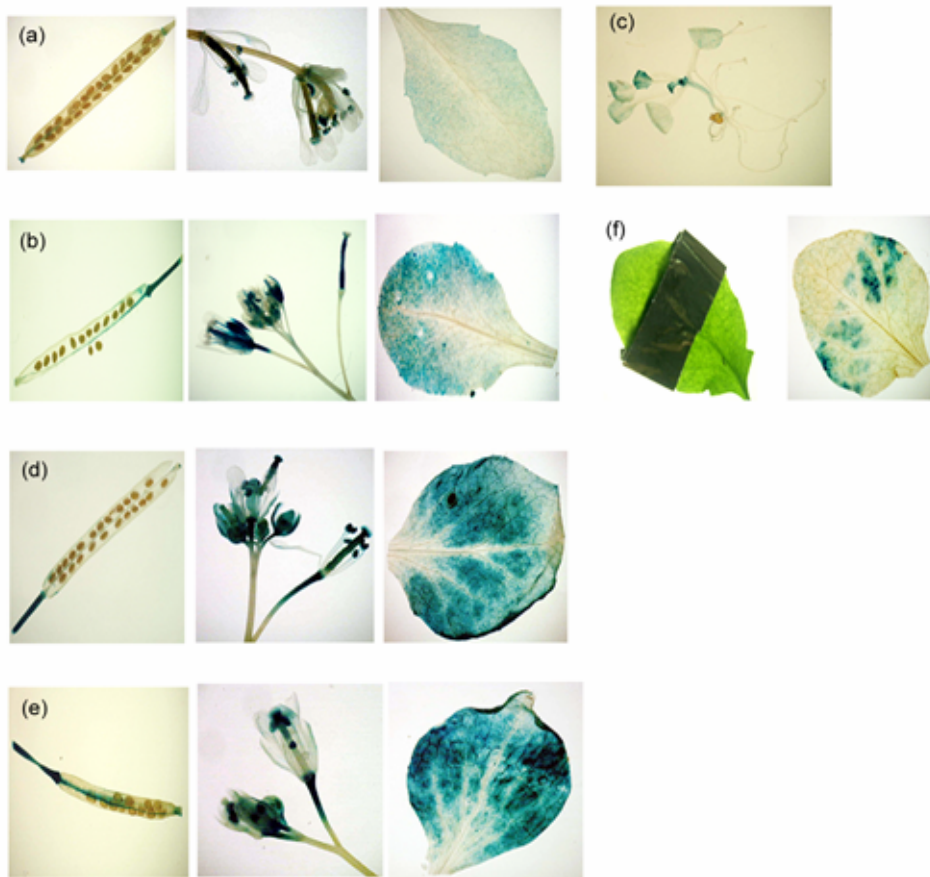
Figure 2-11. GUS activity in transgenic plants. (a) Silique and seed (left), flower (middle), mature rosette leaf (right) under normal condition (16 h light/ 8 h dark). (b) silique and seed (left), flower (middle), mature rosette leaf (right) treated with sugar starvation for 1 day. (c) 10-day-old transgenic seedling treated with sugar starvation for 1 day. (d) silique and seed (left), flower (middle), mature rosette leaf (right) treated with darkness for 1 day. (e) silique and seed (left), flower (middle), mature rosette leaf (right) treated with darkness for 2 days. (f) treatment with partial darkness for 1 day; before (left) or after (right) treatment.
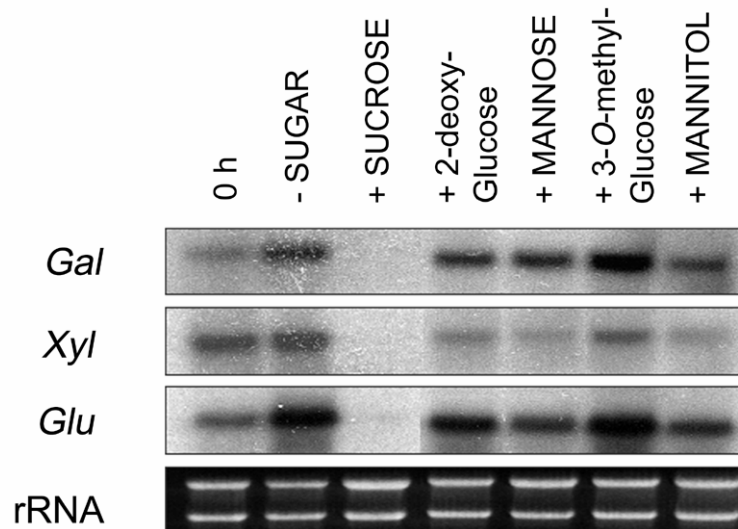
Figure 2-12. Effects of glucose analogs on transcript accumulation. Seven-day-old cells were rinsed and transferred to a medium containing indicated glucose analogs (30 mM 2-deoxy-glucose, 90 mM for other glucose analogs), or no sugar, and after 48 h, transcript level were estimated by RNA gel-blot hybridization. RNA isolated from non-treated cells (0 h) was used as the control. A 5-μg of total RNA was loaded per lane, were subjected to hybridization with indicated probe. rRNA was visualized with ethidium bromide staining for the loading control.

PROMOTER ANALYSIS OF A GENE ENCODING PROTEIN KINASE REGULTATED BY SUCROSE

Sugars act as a signal for activation/repression of sugar metabolic pathway. To studies on mechanism of sugar sensing and signaling in plants, metabolites, transporters, enzymes, transcription factors and hormones were suggested to be required for sensing and signaling. Major achievements were obtained from genetic approaches using *Arabidopsis* by screening mutants, which showed different response to sugar in germination medium (Sheen *et al*., 1999; Gibson, 2000; Smeekens, 2000; Rook *et al*., 2001). These experimental results indicated the importance of hexokinase, which corss-talks with plant hormones such as ABA (Ishitani *et al*., 1997; Léon and Sheen, 2003). Nevertheless, molecular mechanism of sugar signaling has not been fully understood.

In this Chapter, I analyzed the promoter of a gene encoding an *Arabidopsis* protein kinase (AtSR2) regulated by sucrose. Transcripts of AtSR2 were induced by sucrose, high salinity and cold. Since AtSR2 appeared to be a typical sugar-responsive protein, I tried to identify regulatory elements, which are responsive to sugar as well as other environmental stimuli.

RESULTS

Transcripts of AtSR2 were induced by sugar, salt and cold

Expression profile of *AtSR2* in response to various stimuli was examined by RNA gel-blot analysis. Transcript level increased in leaves 79 h after the onset of treatment with 1% sucrose, and gradually decreased when sucrose was depleted. Re-supply of sucrose resumed transcripts within 7 h (Fig. 3-1a). In addition to sucrose, transcripts of *AtSR2* were also induced when dark-adapted leaves were exposed to cold and salt stress (Fig. 3-1b).

Transcriptional starting site was determined

The transcriptional starting site of *AtSR2* was determined by RT-PCR analysis. Results suggested that it located between (–74) GAGACACTCACCAAACAACTCGTCC (–54) and (–42) CTTCAGAAAACTCAGGCACCATTTT (–18) from the translational starting site. From this information and consensus sequence for transcriptional starting site in plants, transcriptional starting site was predicted to be located at AACC located at -49 bp from the translational starting site (Fig. 3-2a). A putative TATA box was located at –41 to –38 bp from the predicted transcriptional starting site (Fig. 3-2a). A 1793 nucleotides sequence from this point was regarded as the *AtSR2* promoter, and further analyzed.

Sucrose and other stresses required different responsive elements

The *AtSR2* promoter was fused with luciferase reporter gene and introduced into *Arabidopsis*. T_2 plants from independent plants were used for luciferase assay. When the 1793

bp promoter was fused with luciferase, activity was clearly induced by sucrose and other stresses. Subsequently, a series of deletion construct fused with luciferase was subjected for assay. Induction by sucrose was still observed when promoter was deleted up to 183 bp, indicating the existence of sucrose-responsive elements in this region (Fig. 3-3). This region contained two sequences, (−72) GAATAATAAAAAAGCAA (−56) and (−170) GAATTAATTACAAAATAATG (-141), which are found in promoter regions of sporamin, patatin, sucrose synthase and β-amylase. They were pointed to be important for sucrose-induced expression (Fig. 3-2b). In contrast, induction by cold and NaCl dismissed, when the promoter was deleted to 553 bp. Since the 693 bp was still active, the positive element for cold and NaCl was asigned to reside between −693 and −553 bp (Fig. 3-3).

Expression of AtSR2 is tissue specific

The 1793 bp of *AtSR2* promoter was fused to β-Glucuronidase (*GUS*) reporter gene, introduced in *Arabidopsis*, and examined for tissue-specific expression. The promoter was active in imbibed seeds (Fig. 3-4a). In young seedlings 2 days after germination, GUS staining was observed in embryonic axis (Fig. 3-4b). At 10 days after germination, expression in embryonic axis still persisted and roots were also stained (Fig.3-4c). In mature plants, staining was mainly observed in leaf-veins and peduncle (Fig. 3-4d). In flowers, GUS expression was found in the vascular bundle of the stamen filament and at stigma, where

filament joins the pistil (Fig. 3-4e). In addition, vascular specific expression was observed in cross-sections of roots (Fig. 3-4f). When mature leaves were treated with sucrose, staining became intensive, indicating GUS activity was induced (Fig. 3-4g).

DISCUSSION

Many plant genes are regulated by sugars at transcriptional level. The *cis*-element in promoters of these genes has also been identified (Grierson *et al*., 1994; Ishiguro and Nakamura, 1994; Maeo *et al*., 2001; Giuliano *et al*., 1988; Zourelidou *et al*., 2002). However, so far identified *cis*-elements are not necessarily conserved among them, suggesting each gene to be specifically regulated by its own promoter. In order to understand such specificity of the promoter, I analyzed the promoter activity of a gene encoding a protein kinase, AtSR2 whose transcription is regulated by sucrose, as the model system. Experiments with a series of deletion constructs showed that a 183 bp from the putative transcription initiation site conferred response to sucrose. In this region, there are two sequences, (−72) GAATAATAAAAAAGCAA (−56) and (−170) GAATTAATTACAAAATAATG (−141), which are similar to *cis*-sequences for sugar induction in patatin, sucrose synthase and β-amylase (Fig. 3-2b). This suggests the presence of a common *cis*-element in some sugar-responsive gene, although their function remains to be determined.

During this study, I found that *AtSR2* transcripts accumulated upon not only sucrose supply, but also cold and NaCl stresses. The promoter assay using luciferase activity indicated that transcription was dismissed when promoter was deleted to −553 bp. Since induction was observed with -693 bp promoter, the *cis*-element that drives expression of *AtSR2* was suggested to locate at the region between −693 and −553 bp. This points to that *cis*-elements for cold and salinity are independent from those for sucrose. Since ABA often plays a role in signaling pathways of cold and salt, and since ABA cross-talks with sugar sensing (Gazzarrini and McCourt, 2001), I examined the relationship between the two. However, my experiments indicated no correlation between transcriptional regulation by sugars and salt or cold response.

The *AtSR2* was found to be located predominantly vascular tissues. This expression pattern may reflect a high concentration of sucrose in these tissues. However, sucrose supply did not induce GUS expression in other tissues, suggesting that the *AtSR2* promoter contains additional element for tissue specific expression in addition to sugar responsive element.

METRIALS AND METHODS

Construction of AtSR2 promoter-b-glucuronidase(GUS) transcription fusions

The *AtSR2* promoter region (1793 bp) was isolated by PCR from *A. thaliana* genomic DNA

using a primer set of 5'-CTGCAGTCTAGAAATTGAACTTTTGTTTTTAACACC-3' and 5'-CCCGGGTCCATGGCGGCGGCGGAAGG-3', which contain an *XbaI* and a *SmaI* site, respectively. The resulting PCR product was cloned into pBI101 vector by digestion with *XabI* and *SmaI*, and sequenced.

Construction of AtSR2 promoter-luciferase (LUC) transcription fusions

Five types of promoter-LUC transcription fusions were constructed by fusing the respective length of promoter region with the LUC reporter gene in the pBI101 vector. To make a series of 5'deletion constructs, the *AtSR2* promoter fragment was amplified by PCR using designed primers. Deleted promoter fragments were excised as *XbaI*/*NcoI* and cloned into *AvrI*/*NcoI* site of LUC expression vector pSP LUC$^+$. pBI 101 DNA was digested with *SacI* and blunt ended by Klenow fill-in DNA synthesis, and ligated to *XhoI* linkers. After allowing the modified pBI 101 vector to self-ligate, all deletion derivatives of fusion constructs were digested with *BglII*/*XhoI* and subcloned into the modified pBI 101 vector at *BamHI*/*XhoI* site.

Transgenic plants, and growth conditions

Each construct described above was transformed into *Agrobacterium tumefaciens* stain C58, which was used to transform *Arabidopsis thaliana* (ecotype Columbia) by floral vacuum infiltration (Clough and Bent, 1998; modified from Bechtold *et al*., 1993). Transformants were selected on 1/2 Murashige and Skoog medium supplemented with 1 % sucrose, 0.9 %

agar, and 25 µg/mL kanamycin. Several independent transformants from each group were planted in soil and maintained at 22   under a 16 h light/8 h dark cycle, self-fertilized, and T$_2$ progeny was obtained. Individual T$_2$ was used for luminescence assays and GUS activity.

Luciferase assay and histochemical localization of GUS activity

Luciferase assays *in vitro* were performed according to the manufacturer's instructions (Promega E1500) with a luminometer. LUC activity was measured over a 5s period. Protein concentration was determined by Bradford reagent (BioRad) with BSA as a standard. To determine the localization of the enzyme acitivity of the GUS gene, tissue samples were stained with 5-bromo-4-chloro-3-indolyl β-glucuronide (X-Gluc) solution as described by Yang *et al*. (2002). After detection of blue color, chlorophyll was extracted using a series of 50-100% ethanol.

Sugar, cold, and salt treatment

For Northern analysis, leaves from wild type *Arabidopsis* plants were treated without sugar (water) under darkness at 28   for 1 day and then transferred to water containing 1 % (w/v) sucrose, 200 mM NaCl at 28   or water at 4   and incubated for 1 day in darkness. For luciferase assay, leaves of cultured transgenic plants carrying various fusion genes were incubated on water without sugar for 1 day in darkness and then treated with 1 % (w/v) sucrose, 200 mM NaCl at 28   , or water at 4   in darkness for 1 day. As a control, leaves

were placed on water without sucrose at 28   in darkness for 1 day.

RNA isolation and northern-blot analysis

Isolation of RNA and northern-blot analysis were performed as described previously (Chapter 1). Total RNA was extracted from *Arabidopsis* leaves, and aliquots of 5-μg of total RNA were denatured, fractionated by eletrophoresis on a formaldehyde-agrose gel, blotted onto Hybond-N nylon membrane (Amersham-Pharmacia) (Suzuki *et al*., 2001). Membrane was subjected to hybridization using ^{32}P-labelled cDNA probes, which prepared by from full-length of *AtSR2* gene.

Reverse transcription-PCR

A 250 ng of RNA from different tissues was used as templates with different forward primers 5'-AAGATTCTACTTCTCAGTGACAGCA-3' (-132 bp) 5'-CAAGGGTAGTTTCG-GAATAATAAAA-3' (-94 bp), 5-GAGACACTCACCAAACAACTCGTCC-3' (-36 bp), or 5'-CTTCAGAAAACTCAGGCACCATTTT-3' (+1 bp) and common reverse primer 5'-CCGTTTTGTAGATGCTCCGGTAAAG-3' (554 bp). After reverse transcription (70   for 10 min) PCR was carried out under following conditions: 96   for 2 min; 27 cycles of 95 for 30 s, 55   for 30 s, and 72   for 30 s; followed by further extension at 72   for 7 min.
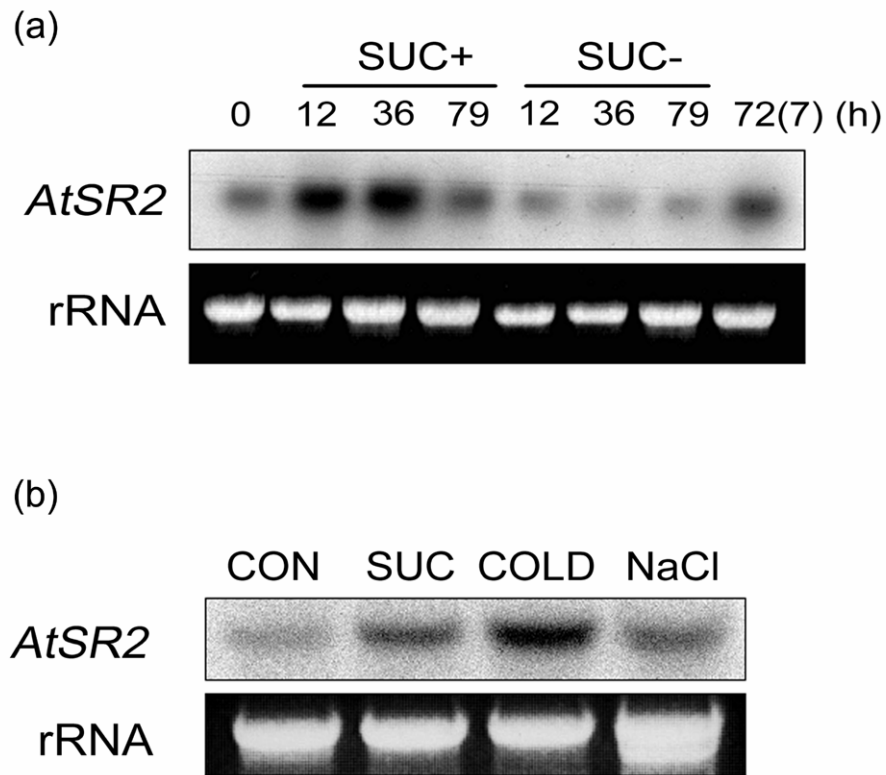
Figure 3-1. RNA gel-blot analysis of *AtSR2* transcripts. (a) Leaves from 3-week-old *Arabidopsis* plants were transferred to a sucrose-free or sucrose-containing water and further incubated under the dark for up to 79 h. Leaves were supplied with sugar by transferring from sucrose-free water for 72 h to water supplemented with 1 % sucrose (w/v) for 7 h. (b) Leaves from 3-week-old *Arabidopsis* plants were transferred to solution containing with 1 % (w/v) sucrose, 200 mM NaCl at 28   , or water at 4   for 48 h. Total RNAs were extracted from each sample treated with sucrose, cold, and NaCl, respectively. Each lane was loaded with 5-μg of total RNA.

(a)

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GACTGATGAATATTGGTGAGTTCTAGTGTGTGACAAAGATGCCATTGTTGACTAGCAAAT        -1819
TCTAAATCTGAGGAGAATAAAATAATTCTAGAAATTGAACTTTTGTTTTTAACACCAAAA        -1759
TCAAAACCAATGGGCCCAATTCTTTGCTGACTTGGCCAAATGAGAGCCGTCCATATGTCA        -1699
GTCGACAACTGCCAAACAATCTTGACGGTTTGTTAGTTTGGCTCAAAAGCCACCAGTAAT        -1639
CACTGATCAGTTTGGGACTTGAGTAAATGACTTATTTACCCTTAGTTCAATAATACGGTT        -1579
TTGTGTGATTTAATTTGCTAGTTAAGTTAAACCATATTTAAGGATGGTTTAATTACATTT        -1519
CAGGTGGAAAAAATTGATGAGAGAATCTAGCTAGAAAAAGATGGACACGTATGGACCGTT        -1459
GAAAGTAGGAATACAAATTATTTAAGGAATACAATATTTAATTTCAACACCAAGTGTCGA        -1399
ATAGATACAACAATAATAATGATGAACACCAAGATTTATTTTATATTCTCTATTTTGAAT        -1339
ATTGATTACTCCAACTTTTTCATGATTTGTTTTTCAGCACTTGAGATTGGAATAGACACA        -1279
CTGTGATATCGACCATAAAATATTTTCTTAGGAATATATCGTTGTGTATAAAGTATAAAG        -1219
TTAAGTGTAATGCTTTAAACAAGAAAAATAAAAGAGTTGAGATTGTAATCATAGCAAAAG        -1159
GCTATTTTATAGACCTTTTAGCGGATGGAAAAAAAAAAAAAAAAACACCTTTTAGCTACA        -1099
TGATGTAGCCTACATCATATATAAAAAAGATAATAGAATTATTTAAGAAATGTAATGTGT        -1039
TATAAATTAATAACCAACACATACACAATAAGATTCATATAAGTTTATGAAATCAAGGTT         -979
GTGATATTGTTGTTATTGAGATCCAAAACTTAAAATCCAAAATATCAGAACTTTTGGAGC         -919
ATTGGATTATATAAAATATATTTTTCTCAATCAAATCTTTTTTTTAGTTAACAACTTTAAA        -859
TTAATCGCAATAGAATTATTCATGAAAAATGTTATTTACATATAACCCAATCCAATGATT         -799
TCAAAAGAGATATTATAGAATTTTGTAAAGCATTAAAGATTTAACTACATTTTGTTACAT         -739
AGACCCATAATCATAAAAACCTTTGATTCATAGTTTCATTCTTATAATGGTCTCTTAAGTA        -679
TGAAGGTGTTGTAGCGGAGACTAGAAATATATATGAGGGTAATGTTTGTCTGTTGAGACA         -619
CATTTTTACAAGCTATTAAAAAAAAAATGATTCCAACAATATTTATTGAAAGTAAATGTATT        -559
TTATTAGGAGATTTTAAAATTAATGTCATATTCTAAAATGTACAATATATACATTATTTT         -499
TTGAAGATTTTACAATAAATTTCAAAGTGAAACTAAAAATGATTTTATTAAATTTATATAC        -439
ATAACTCTTTGTGTTTTTTTGACAGTCATATATATAACTCTTAAACACAAGCTTTTACAG         -379
AACATCTACAGTTATTTTAGGTGAATAAAACACTCCAATTTCAAACAATTTAAATAAATAA        -319
ATGATAATTATCTGTCAACCCATAAACTTTACATTGTAAGACCCATTTTATTTTATTTATT         -259
TTGACTATTTATGGATATGTGATATTATTGAGAAAAAAAAACACTATTACTAATTGACAGA        -199
AAAAATACACTTAATATTTTCTCCTAAATTTTCTCAAC<u>GAATTAATTACAAAATAAT</u>GTG        -139
TATACCAAGTAAAAAGATTCTACTTCTCAGTGACAGCAGAACTAATTTATTCAAGGGTAG         -79
TTTCG<u>GAATAATAAAAAAGCAAT</u>AAATCTCTTTTTTCC<u>TATA</u>AAAGCAAAGAGACACTCAC        -19
CAAACAACTCGTCCCTGAAACCTCTCTTCAGAAAACTCAGGCACCATTTTCTCCTTCCGC          35
                                    →+1
CGCCGCCATGGAATCACTTCCCCAGCCGCAAAACCAATCATCTCCGGCAACAACTCCGGC          95
GAAGATCCTCCTTGGGAAATACGAACTCGGTCGTCGTCTCGGTAGCGGAAGCTTCGCGAA         155
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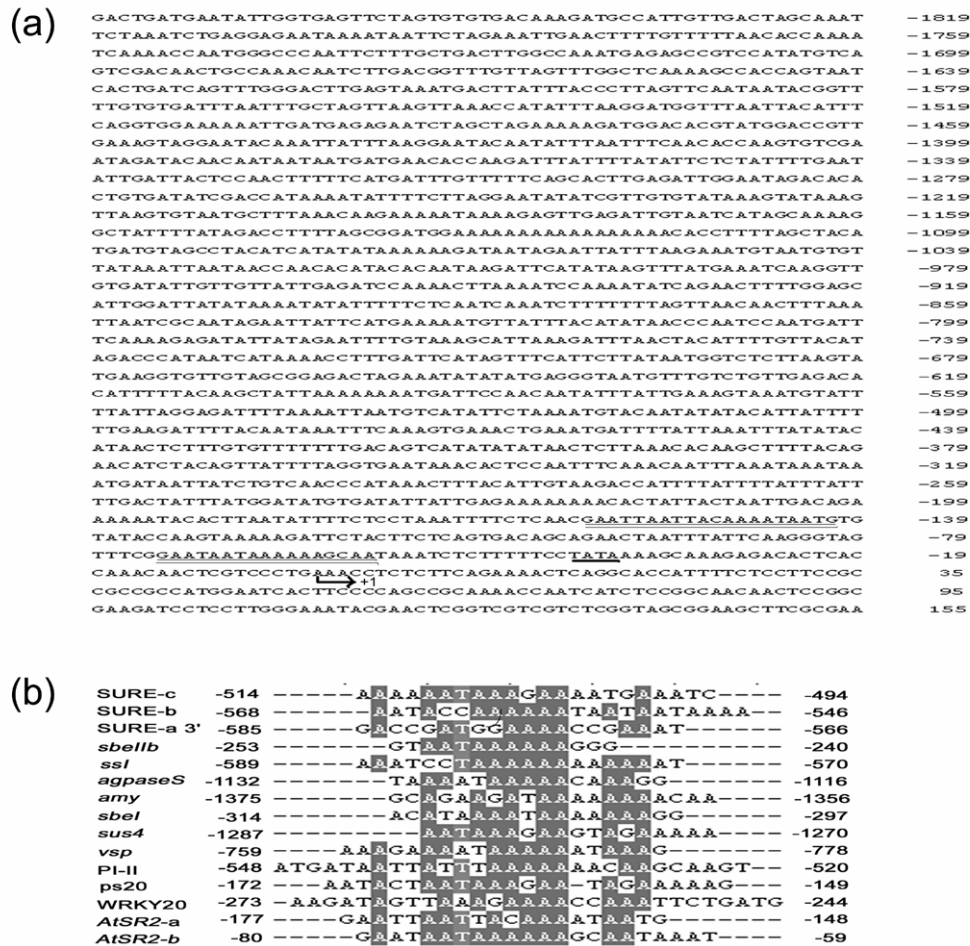
(b)

SURE-c	-514	------AGAAAAATAAAGAAAATGAAATC----	-494
SURE-b	-568	-------AATACCAAAAATAATAATAAAA--	-546
SURE-a 3'	-585	------GACCGATGGAAAACCGAAAT------	-566
sbeIIb	-253	-------GTAATAAAAAAGGG---------	-240
ssI	-589	------AGATCCTAAAAAAAAAAAT------	-570
agpaseS	-1132	-------TAAAATAAAAACAAAGG-------	-1116
amy	-1375	-------GCAGAAGATAAAAAAAACAA----	-1356
sbeI	-314	--------ACATAAAATAAAAAAAAGG-----	-297
sus4	-1287	-------AATAAAGAAGTAGAAAAA----	-1270
vsp	-759	----AAAGAAAATAAAAATAAAG-------	-778
PI-II	-548	ATGATAATTATTTAAAAAAACAAGCAAGT--	-520
ps20	-172	---AATACTAATAAAGAA-TAGAAAAAG---	-149
WRKY20	-273	-AAGATAGTTAAAGAAAACCAAATTCTGATG	-244
AtSR2-a	-177	----GAATTAATTACAAAATAATG-------	-148
AtSR2-b	-80	-----GAATAATAAAAAAGCAATAAAT----	-59

Figure 3-2. Nucleotide sequence of *AtSR2* promoter region. (a) The putative transcription initiation site is numbered as one and predicted TATA sequence is marked with bold line. Double underlined sequences represent sites similar to putative sugar-inducible element in the several plant promoters. (b) Sugar-responsive elements in plant promoters. SURE-c, SURE-b, SURE-a 3' refer to the native or modified SURE sequences of barley *iso1* (Sun *et al.*, 2003); *sbeIIb*, barley *sbeIIb* (Sun *et al.*, 1998); *ssI*, barley *ssI* (Gubler *et al.*, 2000); *agpaseS*, barley *agpaseS* (Thorbjørnsen *et al.*, 2000); *amy*, *Arabidopsis* gene for β-amylase (Mita *et al.*, 1995); *sbeI*, maize *sbeI* (Kim and Guiltinan, 1999); *sus4*, potato gene for sucrose synthase (Fu *et al.*, 1995); *vsp*, soybean gene for vegetative storage protein (Rhee and Staswick, 1992); PI-II, potato gene for proteinase inhibitor II (Kim *et al.*, 1991); ps20, potato patatin class I gene (Grierson *et al.*, 1994); WRKY20, the rice *susba2* ortholog (Sun *et al.*, 2003); *AtSR2*-a, b, *Arabidopsis* gene for SNF1-related protein kinase (this work).
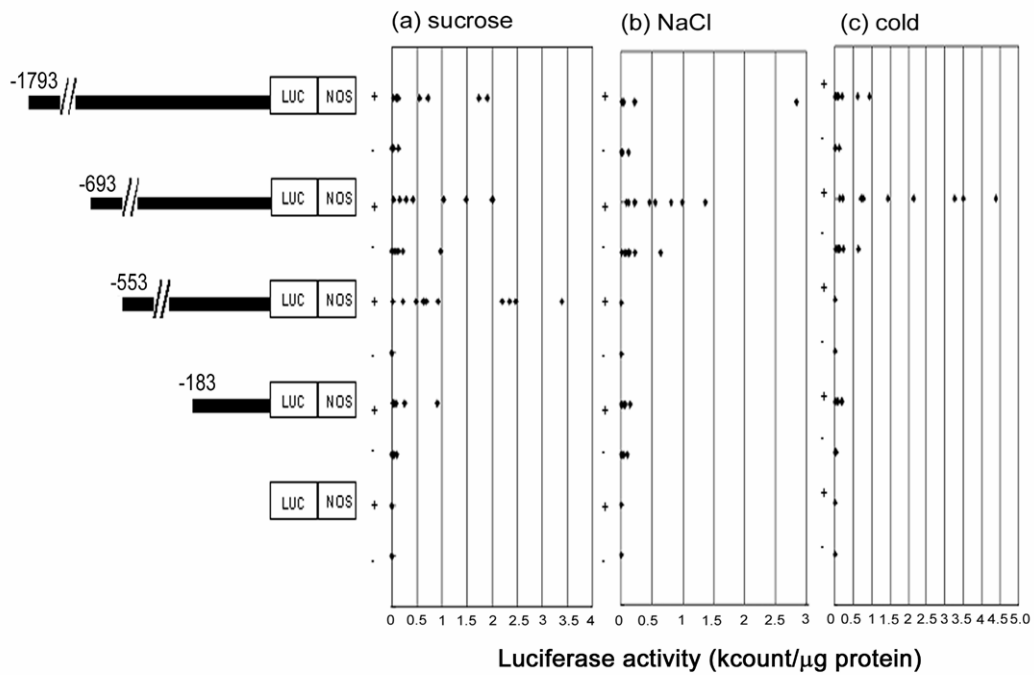
Figure 3-3. Effects of 5'-terminal deletions. The structures of various deletion constructs of *AtSR2* promoter fused to LUC reporter gene are shown on the left and their activity in leaves of T₂ transgenic plants are shown on the right. Leaves from transgenic plants were incubated either in water (indicated with -), or in 1 % sucrose, containing 200 mM NaCl at 28 , or water at 4 for 1 day under darkness (inducated with +). LUC activity represents the values obtained from independent transformant lines.
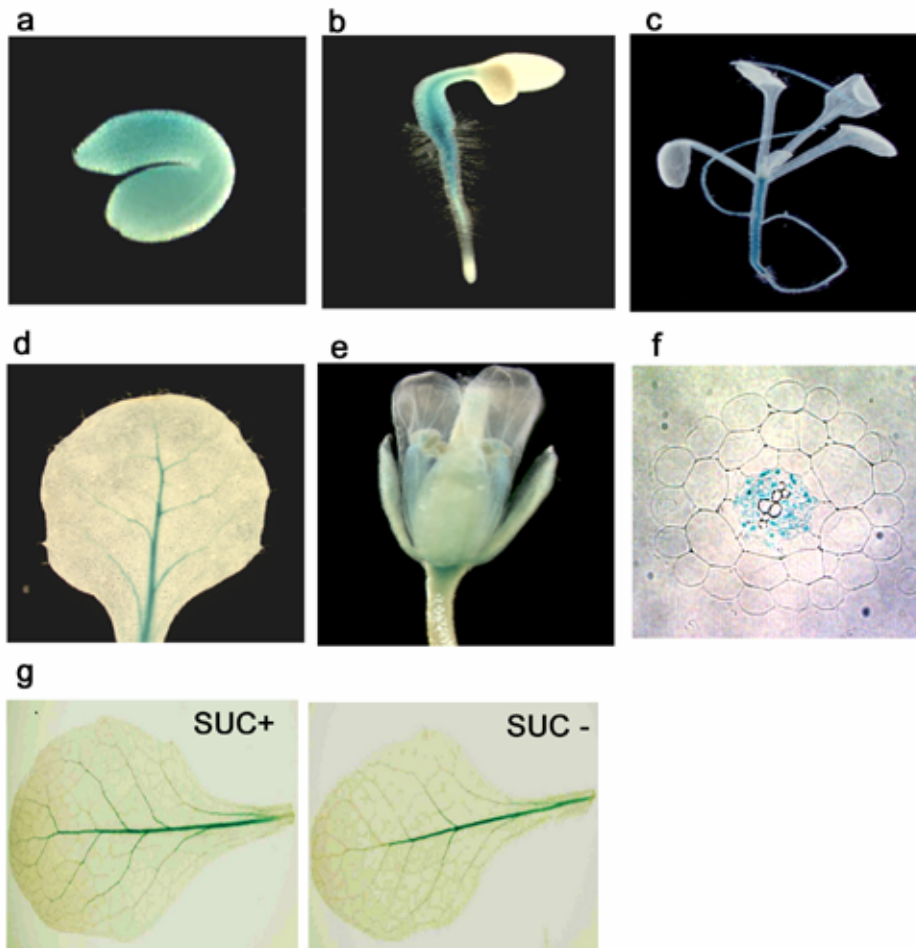
Figure 3-4. Histochemical localization of GUS. (a) imbibed seed, (b) 2-d-old seedling, (c) 10-d-old seedling , (d) mature rosette leaf (e) flower, (f) cross-section of a root, (g) leaf treated with (left) or without (right) 1%(w/v) sucrose.

CONCLUDING REMAERKS

Living organisms on the earth depend on solar energy, which is converted to organic compounds only by plants. This process is photosynthesis. The primary photosynthetic products are sugars, which are subsequently used as energy source as well as structural components of plant cells. Carbohydrates derived from sugars such as starch are also important to feed animals including humans. In spite of the importance, carbohydrate metabolism in plants has not been fully elucidated. In this past decade, sugars have been recognized to act as signal molecules regulating many cellular processes including carbohydrate metabolism. Thus, I thought that identification of genes regulated by sugars would give a new insight for understanding of carbohydrate metabolism in plants.

From this point of view, I initiated my work by screening genes regulated by sugars at transcription level, using a model plant *Arabidopsis*. Identified genes were mainly those encoding enzymes involved in various metabolic pathways including carbohydrate metabolism. Among those, I characterized genes encoding putative β-galactosidase, β-xylosidase, and β-glucosidase, which are predicted to be involved in degradation of cell wall polysaccharides. Their expression apparently induced when plants were kept in the darkness or depleted with sugar components. From this observation, I concluded that these

enzymes produce a new carbon source by catalyzing breakdown of cell wall materials. This idea was supported by experiments, showing them to be secreted in culture medium of suspension cells when sugars were deleted. In addition, galactose, xylose and glucose were utilized as carbon source in suspension cells. Furthermore, I directly demonstrated a decrease of cell wall materials including pectin and hemicellulose I during sugar depletion induced by impaired photosynthesis. Thus, I propose here an idea that one of functions of plant cell wall is to supply carbon source in addition to contribution to cell shape and physical support of plant bodies.

In order to confirm the idea, direct evidence is desirable. For example, analysis of mutants, in which my genes are inactive, will be effective to show a defect of viability under impaired photosynthetic condition. Detailed analyses of sugar composition in cell wall will be helpful. Understanding of the signaling mechanism of the present genes by sugar depletion is also important. My preliminary experiments suggested that they are regulated in hexokinase-independent pathways, which should be analyzed in detail to draw a definitive conclusion.

Overall, I conclude that cell wall is effectively subjected to recycling for nutrient allocation, and therefore show that it plays a much more important role for plant life than hitherto thought.

ACKNOWLEDGEMENT

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LIST OF PUBLICATION

Lee E. –J., Koizumi N., and Sano H. (2004). Identification of genes that are up-regualted in

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