MOLECULAR AND GENETIC DISSECTION OF SUGAR SIGNAL TRANSDUCTION PATHWAY IN ARABIDOPSIS THALIANA

DISSERTATION

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ABSTRACT

While sugars are important signaling molecules for the control of plant growth and development, little is known about the molecular mechanisms underlying sugar sensing and signal transduction in plants. To date, many glucose insensitive mutants have been found to be alleles of abscisic acid (ABA) biosynthetic or perception mutants. This scenario implies that traditional mutant screens may preferentially identify indirect effects rather than components directly involved in sugar response. On the basis of the information on the previous mutant screens, steady-state mRNA analysis, and global transcription profiling, I hypothesized that sugar signals might be transmitted through an ABA-independent signal transduction pathway. I have established a mutant screen system using marker genes that are very sensitive to glucose. *ASN1* (*asparagine synthetase1*) is completely repressed by low levels of exogenous glucose (< 0.1%) in less than 3 h and is derepressed by removing exogenous glucose from the culture medium. Glucose repression of *ASN1* also involves protein phosphorylation but not *de novo* protein synthesis. In addition, hexokinase appears to be required for the glucose repression of *ASN1*. Most critically, *ASN1* repression by glucose is not affected by ABA under the conditions tested.

To carry out a screen for sugar response mutants with altered expression of *ASN1*, wild-type *Arabidopsis* plants have been transformed with reporter constructs carrying the
ASN1 promoter fused with luciferase or green fluorescent protein genes. Homozygous transgenic plants containing the reporter gene have been generated. Significant quantities of EMS (ethyl methane sulphonate) mutagenized M_{2} seeds from three selected lines were obtained. A large-scale mutant screen has been carried out using a luminescence-imaging system. Over 50 putative mutants have been identified. In addition to abnormal ASN1 expression, a high percentage of the mutants display altered growth and development, including large plant size and late flowering. Phenotypic and molecular characterization of putative mutants is expected to reveal signaling mechanisms involved in the regulation of ASN1 expression by sugar.

Recent DNA microarray analyses in our lab have shown that a wide range of genes are regulated by glucose, such as those associated with carbohydrate metabolism, transcriptional regulation, and metabolite transport. Remarkably, genes involved in nitrogen metabolism are largely regulated by sugar rather than inorganic nitrogen, thus revealing an underlying molecular mechanism for the role of carbon/nitrogen ratio in plant growth and development. Among those, I found the expression of two putative ACT (Aspartokinase, Chorismate mutase, and prephenate dehydrogenases (TyrA)) domain-containing protein kinase genes (ACTPK) was highly affected by glucose. In bacteria, ACT domains have been shown to serve as amino acid-binding sites for feedback regulation of metabolic enzymes to be involved in nitrogen metabolism. The role of ACT domain-containing genes in plants is largely unknown. Functional characterization has been accomplished to determine the sugar response and temporal and spatial expression of ACTPK. The ACTPK3 transcript was completely abolished by low levels of exogenous glucose (0.1%, w/v) in 3 h. Interestingly, steady-state mRNA
analysis results showed that \textit{ACTPK3} is likely controlled by the circadian clock that is epistatic of sugar response. The glucose repression of \textit{ACTPK3} required sugar transport, sugar phosphorylation, hexokinase, \textit{de novo} protein synthesis, and protein dephosphorylation. Furthermore, the \textit{ACTPK3} repression by glucose was not modulated by ABA. Several T-DNA knockout lines have been obtained and results of initial phenotypic analysis suggested that \textit{ACTPK} might be involved in plant sugar response.
Dedicated to

My mother

For her great inspiration and encouragement to my science endeavor
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LISTS OF SYMBOLS / ABBREVIATIONS / NOMENCLATURE

3-OMG, 3-\(O\)-methyl glucose

ABA, abscisic acid

\(aba\), ABA deficient

\(abi\), ABA insensitive

ABRC, The Arabidopsis Biological Resource Center (www.arabidopsis.org/abrc)

ACC, 1-aminocyclopropane-1-carboxylate

ACR, ACT domain repeat gene

ACT, aspartokinase, chorismate mutase and prephenate dehydrogenases (TyrA)

ACT-PK, ACT domain-containing protein kinase

AD, arbitrary degenerate primer

AGPase, AGP, ADP-glucose pyrophosphorylase

AK, aspartate kinase

AK/HSD, aspartate kinase-homoserine dehydrogenase

ALS, acetolactate synthase

Amy, amylase

\(AS\), glutamine-dependent asparagine synthetase gene

AGI, \emph{Arabidopsis} Genome Initiative
AGRIS, Arabidopsis Gene Regulatory Information Server  
(arabidopsis.med.ohio-state.edu)

Asn, asparagine (amino acid)

ASNI, Arabidopsis glutamine-dependent asparagine synthetase gene

At, Arabidopsis thaliana

ATase, adenylyl transferase

BA, 6-benzyladenine

bp, base pair

BLAST, basic local alignment search tool

bZIP, basic leucine zipper domain

CAB1, chlorophyll a/b-binding protein

CaMV, cauliflower mosaic virus

CCD, charge-coupled device

CDPK, calcium dependent protein kinase

CHX, cycloheximide

ctr, constitutive triple response

DCMU, 3-(3,4-dichlorophenyl)-1-1-dimethyl-urea

DF, dilution factor

DIN, dark-inducible genes

dNTPs, deoxynucleotide triphosphates

ein, ethylene insensitive
EMS, ethyl methane sulphonate

EST, expressed sequence tag

eto, ethylene overproducer

etr, ethylene response

GenBank, NIH genetic sequence database

GFP, green-fluorescent protein

gin, glucose-insensitive

GUS, β-glucuronidase

HXK, hexokinase

HXT, hexose transporter gene

isi, impaired sugar induction

LB, left T-DNA border

LUC, luciferase gene

MES, 2-(N-morpholino) ethanesulfonic acid

MS, Murashige and Skoog

MPSS, Massively Parallel Signature Sequencing (mpss.udel.edu/at/java.html)


NTPII, neomycin phosphotransferase coding gene

PC, plastocyanin

PDH, prephenate dehydratase

Pfam, Protein families database (www.sanger.ac.uk/Software/Pfam/)
PGDH, phosphoglycerate dehydrogenase-like protein

PK, protein kinase-like protein

PLACE, plant cis-acting regulatory DNA element (www.dna.affrc.go.jp/htdocs/PLACE)

Pnos, nopaline synthase promoter

PR, Pathogenesis-related

Pro, proline

RAB, responsive to ABA

RB, right T-DNA border

rbcS, ribulose-1,5-bisphosphate carboxylase small subunit

Rgt, Regulation of glucose transporters

RT-PCR, reverse transcription-polymerase chain reaction

sis, sugar insensitive

SNF1, sucrose non-fermenting-1

SnRK, SNF1-related protein kinase

sun, sucrose uncoupled

TAIR, The Arabidopsis Information Resource (www.arabidopsis.org)

T-DNA, transferred DNA

TAIL-PCR, thermal asymmetric interlaced-polymerase chain reaction

TPM, transcripts per million

UTase, uridylyl transferase

WT, wild-type plant(s)
CHAPTER 1

INTRODUCTION

1.1 SUGAR SENSING AND SIGNALING IN EUKARYOTIC CELLS

All organisms have evolved to sustain their lives by utilizing available nutrient sources in natural environments. The cells and tissues of all organisms are constituted of the same basic molecules: carbohydrates, lipids, proteins, and nucleic acids. Each of these molecules also participates in a variety of cellular functions. For example, carbohydrates are used as energy storage, metabolic intermediates, and structural framework for RNA and DNA. In addition to metabolic and structural functions, sugar (i.e. glucose) also plays an important role by affecting the expression of numerous genes. Although sugar sensing and signaling have been studied extensively in diverse organisms, the molecular mechanisms are largely unclear, particularly in higher plants.
1.1.1 *Sugar sensing and signaling in yeast and mammals*

Like most organisms, yeast (*Saccharomyces cerevisiae*) prefers glucose as a carbon source. While yeast can utilize a wide range of fermentable and non-fermentable carbon sources, genes involved in alternative carbon usage, respiration, and gluconeogenesis are repressed in the presence of glucose. By contrast, genes encoding glycolytic enzyme, ribosomal proteins, and low-affinity glucose transporters are induced by glucose (Johnston, 1999; Rolland et al., 2001). These selective and rapid gene expression changes enable yeast to survive adverse fluctuation of nutrient sources. Among various glucose-regulated genes, hexose transporter-like genes were found to be critical in glucose sensing and signaling in yeast (Gancedo, 1998; Ozcan and Johnston, 1999). A number of yeast hexose transporters (HXTs) are transcriptionally regulated by multiple glucose signaling pathways (Ozcan and Johnston, 1999). At least 16 of 48 carbohydrate transporter-like genes have been demonstrated to have transport functions. Among the rest, Snf3 and Rgt2, have been identified as sugar sensors that can bind to glucose but are unable to transport glucose. Upon binding to glucose, the cytosolic, C-terminus portions of Snf3 and Rgt2 interact with downstream signaling components, initiating a signaling cascade and ultimately causing the activation of hexose transporters. This glucose-mediated transcriptional regulation controls sugar uptake in yeast. In the absence of glucose, Rgt1 works as a transcriptional repressor and the association of Rgt1 with HXT promoter results in the repression of HXT gene. In contrast, in the presence of high glucose, glucose prevents the association of Rgt1 with HXT promoters thereby
alleviating the repression (Flick et al., 2003).

Microarray analyses using mammalian cells indicated that glucose also has profound effects on the expression of various genes involved in energy metabolism, regulation of insulin biosynthesis and secretion, membrane transport, intracellular signaling, and protein synthesis/degradation (Schuit et al., 2002; Korke et al., 2004).

In mammals, blood glucose concentration is tightly regulated by interplay of various mechanisms in the liver, muscle, and adipose tissues. In the feasting state, these tissues increase the rate of glucose utilization, whereas during fasting they release glucose to the blood from glycogen stores or newly synthesized gluconeogenesis reaction. Hormones, mostly insulin and glucagons, tightly regulate glucose homeostasis. To prevent development of either hyperglycemia or hypoglycemia, pancreatic, b- and a-cells can directly sense blood glucose concentration by secreting insulin or glucagons, respectively (Matschinsky, 1996; Matschinsky et al., 1998). In the b-cells, glucose phosphorylating enzyme glucokinase serves as glucose sensor (Efrat et al., 1994). In addition to glucokinase, glucose transporter (GLUT2) is required to measure the changes in extracellular glucose concentration (Newgard and McGarry, 1995). Recent studies using GLUT2 gene knockout mice showed that the inactivation of GLUT2 leads to a loss of glucose sensing and impaired insulin secretion (Guillam et al., 1997; Guillam et al., 2000; Thorens et al., 2000; Thorens, 2003).
1.1.2 Sugar sensing and signaling in plants

As sessile organisms, plants face many environmental stresses such as changing temperature and fluctuation of light, water, and nutrients. Adapting to these environmental changes, highly flexible carbohydrate metabolic systems have been evolved. While extensive studies on plant metabolism, photosynthesis, growth and development have elucidated the regulatory effects of sugars, the role of sugars as signaling molecules is only beginning to be appreciated.

1.1.2.1 Sugar responses

1.1.2.1.1 Growth and development

Sugars affect plant growth and development directly by providing energy and indirectly by modulating other plant signal transduction pathways such as light, hormone, and nitrogen signaling (Smeekens, 2000; Coruzzi and Zhou, 2001; Leon and Sheen, 2003; Gibson, 2004). Known examples include the role of sugar in cell cycle regulation (Riou-Khamlichi et al., 2000; Lorenz et al., 2003), seed germination (Pego et al., 1999; Price et al., 2003; Dekkers et al., 2004), cotyledon differentiation during embryogenesis (Borisjuk et al., 2002), flowering time (Ohto et al., 2001), and senescence (Dai et al., 1999; Fujiki et al., 2001; Pourtau et al., 2004). Low concentrations of glucose affect seed germination, suggesting an effect independent of osmotic stress. In addition, application of metabolizable sugars relieved the inhibitory effect of ABA on germination but not on seedling growth (GarciaRubio et al., 1997; Finkelstein and Lynch, 2000a; Price et al., 2003). This illustrates that the interactions between sugar and other signaling pathways are complex and may not be additive effects.
1.1.2.1.2 *Metabolism*

Plant carbohydrate metabolism is unique due to the characteristics of sugar production via photosynthesis, sink-source partition, and cell wall synthesis. Through photosynthesis, plants fix carbon from CO$_2$ and supply to leaf cells as energy reserve or to convert simple molecules into complex organic compounds. Therefore, carbon metabolism and signaling are tightly regulated by light. Investigation of carbon and light signal interaction at a genome-wide level in *Arabidopsis* found a number of genes involved in nitrogen assimilation are also controlled by light and carbon (Thum et al., 2003).

*Sugar metabolism*

Sugars are starting materials for numerous biosynthetic processes involved in the production of nucleic acid, proteins, lipids, and polysaccharides as well as coenzymes and numerous secondary metabolites. In addition to being used as metabolic substrates, sugars are also employed as non-metabolic substrates in the signaling pathways for the regulation of gene expression. Because sugar responsive genes have both direct and indirect roles in sugar metabolism, gene expression data have used as an index to link a particular group of genes and their sugar responses with a particular metabolic function. In general, low sugar status enhances expression of genes for photosynthesis, remobilization, and export processes, whereas abundant sugar resources genes for polysaccharide biosynthesis, storage, respiration, and utilization (Koch, 1996).
**Interaction with light**

Carbohydrates are produced through photosynthesis. Light directly or indirectly affects plant sugar metabolism (Ohto et al., 2001). During photosynthesis and carbohydrate production metabolic feedback regulation of photosynthesis determined by the rate of consumption of the end product such as sucrose, starch, and amino acids. In contrast, light regulates the expression of genes for photosynthesis to ensure efficient light use (Paul and Pellny, 2003). An abundant carbon level induces the expression of genes for enzymes involved in the utilization and storage of carbon and can repress genes for photosynthesis. A poor carbon level results in the opposite effect (Koch, 1996).

Although the underlying molecular mechanisms are not clear, interactions between sugar- and light-signaling pathways exist in plants (Berger et al., 1995; Brocard-Gifford et al., 2003).

Furthermore, photosynthesis can compensate for the absence of the glyoxylate cycle during postgerminative growth, and the provision of exogenous sugars can overcome this growth deficiency (Eastmond et al., 2000). Recent studies on carbon/light interaction using genes involved in nitrogen assimilation indicated that carbon/nitrogen signaling depends not only on the light qualities (white, blue, red, and far-red), but also on the fluence (low or high). For example, transcripts of *ASN1* in light-grown plants were not suppressed by an application of lower fluence (2 µE m⁻² s⁻¹) of far-red light in the absence of sugar, but suppressed by the a higher fluence (100 µE m⁻² s⁻¹). Interestingly, in the presence of sugar, higher fluence far-red light slightly relieved the carbon suppression of *ASN1* (Thum et al., 2003). This result implicates light qualities also
affected gene expression by the interaction with carbon.

Research on circadian clock using DNA microarray in *Arabidopsis* suggested that light could play an important role in allocation of assimilated sugars for metabolic usage, transport, or storage (Harmer et al., 2000). Recent microarray analysis on the effects of sucrose and light revealed that over-represented genes in the population of genes regulated by carbon and/or light were associated with metabolism of starch, amino-acid, lipid, fatty-acid, and isoprenoids (Thum et al., 2004). Results indicated a large number of genes are influenced by cross regulation of light and carbon (about 40 %), independent light or carbon signal (about 20%), or both light and carbon together (about 1.6%) (not regulated by either signal alone).

**Interaction with nitrogen**

Carbon and nitrogen metabolism are tightly linked in plants. Sugars regulate many genes involved in nitrogen metabolism and storage. Among various sugar inducible genes are those associated with nitrate assimilation such as nitrate reductase and genes involved in the high (*NRT2*) and low (*NRT1*) affinity nitrate uptake (Cheng et al., 1992; Lejay et al., 1999). On the other hand, exogenous sugars repress other nitrate metabolism-associated genes such as the glutamine dependent asparagine synthetase gene (*ASN1*) (Lam et al., 1994). Carbohydrate to nitrogen (C/N) ratio plays a central and interactive role in regulating seedling growth and development, metabolism, and gene expression (Martin et al., 2002). C/N ratio affects plant morphology, growth rate, storage reserve mobilization, chlorophyll levels, and photosynthetic gene expression. Interestingly, recent transcriptional profiling reveals that exogenous glucose is more
effective than exogenous inorganic nitrogen in affecting genes associated with nitrogen assimilation and amino acid metabolism (Price et al., 2004).

1.1.2.1.3 Stress

Growing evidence supports the role of sugars in diverse stress responses during flooding, pathogen attack, and adverse conditions that trigger early senescence. Flooding (oxygen deprivation) causes an increase in glycolytic fermentation and generates lactate and alcohol. Pathogenesis-related (PR) protein-coding genes in Arabidopsis were activated in leaves when plants were grown in the presence of exogenous sucrose (Thibaud et al., 2004). In addition, antifungal proteins were accumulated in the presence of glucose during berry ripening (Salzman et al., 1998). Delayed senescence has been observed in Arabidopsis HXK1 antisense plants (Xiao et al., 2000). Likewise, transgenic tomato plants overexpressing the AtHXK1 showed that hexokinase is involved in sensing endogenous levels of sugars in photosynthetic tissues and that it participates in the regulation of senescence, photosynthesis, and growth (Dai et al., 1999). In addition, transcripts of dark-inducible genes (DIN) accumulated in senescence leaves of light-grown plant (Fujiki et al., 2001). This result suggests that expression of these light-regulated genes depend partly on cellular sugar levels because sugar levels are low in senescencing leaves. In general, under stress conditions, cells use sugars to fulfill the energy and carbon requirements needed for proper response to stresses. For example the expression of α-amylases is induced in barley leaves by water stress (Jacobsen et al., 1986), in tobacco leaves by virus infection (Heitz et al., 1991), and in mung bean cotyledons by wounding (Koizuka et al., 1995).
1.1.2.1.4 Gene expression

In general, carbohydrate depletion in cells upregulates genes for photosynthesis and carbohydrate remobilization and export, whereas abundant sugars enhance expression of genes for storage and utilization (Koch, 1996). DNA microarray enables genome-wide analysis of gene expression in a quantitative and high throughput way. Coupling gene expression profiling with metabolite profiling will allow modeling of cellular mechanisms involved in metabolic adaptation to a wide variety of growth conditions. DNA microarray analysis in our lab has revealed that glucose regulated a large number of genes, including genes associated with carbohydrate metabolism, signal transduction, metabolite transport, and stress response (Price et al., 2004). A large number of glucose-responsive transcription factors have also been identified and, intriguingly, the majority of them are sugar-repressible. Results from other microarray analyses showed that many sugar metabolic genes are regulated by nitrate or ethylene, suggesting the existence of coordinated interactions on transcriptional levels between sugar and other hormonal pathways (Wang et al., 2003; Zhong and Burns, 2003).

1.1.2.2. Sugar sensing mechanisms

Multiple sugar sensing mechanisms have been found in plants. Although hexose sensing via hexokinase has been demonstrated, other studies suggest that distinct sucrose and trehalose sensing and signaling pathways also exist (Chiou and Bush, 1998; Goddijn and Smeekens, 1998; Rolland et al., 2002; Halford et al., 2003; Schluepmann et al., 2003).
1.1.2.2.1 Sucrose sensing

Sucrose is a major product of photosynthesis in green leaves and serves as the principal long-distance transport compound in most plants. In the past, sucrose sensing in plants were presumed, it was perceived through hexose derived from sucrose hydrolysis instead of sucrose itself (Smeekens and Rook, 1997). However, the expression of the Arabidopsis bZIP transcription factor ATB2 gene was specifically repressed by sucrose (Rook et al., 1998). In vitro transport assay using plasma membrane vesicles derived from detached, sugar-fed sugar beet leaves indicated that sucrose transport significantly inhibited by exogenous sucrose but not by glucose. The expression of the proton–sucrose symporter gene was repressed by increasing sucrose concentrations and paralleled decreased transport activity (Chiou and Bush, 1998). Furthermore, tomato ODC (ornithine decarboxylase) gene accumulated at higher levels in the presence of sucrose but not in the presence of glucose or fructose (Kwak and Lee, 2001). Together, these results suggest the existence of the sucrose-specific response signaling in plants.

1.1.2.2.2 Hexose sensing

Free hexose is produced by the hydrolysis of sucrose or starch. Through ATP-dependent phosphorylation by hexokinase, these free hexoses are converted to the metabolic intermediates such as glucose 6-phosphate, glucose 1-phosphate, and fructose 6-phosphate. Arabidopsis contains at least six hexokinase homologues, i.e. two HXK and four HXK-like genes (Jang et al., 1997; Dai et al., 1999; Moore et al., 2003). Using the protoplast transient expression system, it was found that both sugar transport across the
plasma membrane and hexose phosphorylation by HXK are required to trigger change in
gene expression (Jang and Sheen, 1994). For example, glucose, which is an efficient
substrate of HXK, repressed photosynthetic gene expression. In contrast, non-substrates
of HXK, 6-deoxyglucose and 3-O-methyl-glucose and non-transportable sugars, L-
glucose and sugar phosphates did not cause significant repression of photosynthetic gene
expression. However, mannose and 2-deoxyglucose, which can be phosphorylated by
HXK but inhibit glucose-6-phosphate and ATP production (Klein et al., 1998), caused
repression of photosynthetic gene expression. In non-green tissue, a cucumber cell
culture system, glyoxylate cycle genes malate synthase (MS) and isocitrate lyase (ICL)
were repressed by the addition of sucrose, glucose, fructose, raffinose, 2-deoxyglucose,
or mannose to the growth medium but not by 3-O-methyl-glucose (Graham et al., 1994).
These results suggested that hexose is specifically sensed by plant cells and sugar
transports across plasma membrane and phosphorylation by HXK appeared to be critical
for sugar signaling. Transgenic Arabidopsis plant overexpressing AtHXK genes are sugar
hypersensitive, whereas underexpressing AtHXK genes are hyposensitive to glucose (Jang
et al., 1997). Analysis of Arabidopsis glucose insensitive2 (gin2) mutants indicated
HXK1 has broad functions in glucose responses, including cell proliferation, root and
inflorescence growth, leaf expansion and senescence, and reproduction (Moore et al.,
2003). The separation of catalytic and signaling functions of hexokinase supports the role
of hexokinase as a signaling protein. On the basis of gene expression analysis in HXK
transgenic plants, three distinct HXK signal transduction pathways were proposed in
Arabidopsis (Xiao et al., 2000).
- **AtHXK1-dependent glucose signaling pathway**

The expression of photosynthetic genes such as *CAB1* (chlorophyll *a/b*-binding protein), *PC* (plastocyanin), and *rbcS* (ribose-1, 5-bisphosphate carboxylase small subunit) genes are mediated by the *AtHXK1*-dependent pathway. While the expression of photosynthetic genes in 35S-*AtHXK1* plant were hypersensitive to exogenous glucose, no repression was observed in 35S-*antiAtHXK1* plants (Xiao et al., 2000). Two yeast *HXK2* misexpressing 35S-*YHXK2* plants also showed no photosynthetic gene repressions by exogenous glucose since a dominant negative effect was observed by elevating HXK catalytic activity (Jang et al., 1997). This data indicated that the regulation of photosynthetic gene expression was mediated through an *AtHXK1*-dependent pathway. Glucose induction of *PR1* and *PR5* expression was higher in 35S-*AtHXK1* plants than in the wild-type plants but lower in 35S-*antiAtHXK1* plants than wild-type plants (Xiao et al., 2000). These results indicated *AtHXK1* were required for PR gene expression. Because *PR1* and *PR5* were expressed to the same extent in plants expressing heterologous yeast Hxk2 genes as in 35S-*AtHXK1*, gene expression is depended on HXK catalytic activity but not the signaling function of *AtHXK1* in this pathway.

- **AtHXK1-independent glucose signaling pathway**

The expression of genes such as *AGPase* (*ADP-glucose pyrophosphorylase*), *CHS* (*chalcone synthase*), *AS1* (*asparagine synthetase1*), and *PAL1* (*phenylalanine ammonialyase1*) mediated in *AtHXK1*-independent pathway in which gene expression is independent of *AtHXK1*. The results showed that *AGPase*, *CHS*, and *PAL1* were
upregulated, whereas AS1 was repressed by glucose (Xiao et al., 2000). The effect of glucose on the expression of these genes was independent of the HXK transgenic plants. An Arabidopssis UGPase (UDP-glucose pyrophosphorylase gene)-encoding gene, Ugp, was up-regulated by sucrose to the excised leaves (Ciereszko et al., 2001). The Ugp expression in wild-type and 35S-antiAtHXK1 plants in comparison with 35S-AtHXK1 plants had similar expressions, suggested that the upregulation of UGPase by sucrose might be mediated through an HXK-independent pathway.

1.1.2.3 Sugar signal transduction

Throughout their life cycles, plants continually sense various external and internal stimuli that they use to modulate their gene expression. Among the stimuli, sugar availability triggers the expression of many genes and thereby many metabolic and developmental processes. The components involved in sugar signal pathways have been well characterized in microorganisms, but largely remains unclear in higher plants. However, biochemical, physiological, and genetic approaches have revealed that plant sugar signaling networks directly or indirectly interacted with hormone signaling pathways. In addition, several intermediates such as Ca$^{2+}$, protein phosphatase, and kinases contribute to sugar signal transduction.

1.1.2.3.1 Components involved in sugar signal transduction

The sugar-inducible expression of genes for sporamin and β-amylase in leaf explants of sweet potatoes and that of a β-glucuronidase-fusion gene, with the promoter of the gene for β-amylase in leaves of tobacco requires Ca$^{2+}$ signaling (Ohto et al.,
1995), and they were inhibited by staurosporin and K-252a, inhibitors of protein kinases (Ohto and Nakamura, 1995). These results suggested that the sugar-inducible expression of genes for sporamin and β-amylase involve Ca\(^{2+}\)-mediated signaling that crosstalk with sugar signaling pathways. Transgenic Arabidopsis plants with increased hexokinase (HXK) expression had a much higher sucrose-dependent level of rab18 mRNA, implying the HXK involvement in the sugar signal pathway. Sucrose-related induction of rab18 (abscisic acid (ABA)-responsive gene) was completely inhibited by okadaic acid (OKA), suggested the involvement of specific protein phosphatase(s) in transduction of the sugar signal (Ciereszko and Kleczkowski, 2002).

In budding yeast, sucrose non-fermenting-1 (SNF1) protein kinase trimetric complex plays an important role in glucose repression signaling pathway. SNF1 is closely related to the calcium dependent protein kinase (CDPK) group, which is also included in the plant calmodulin-like domain protein kinases (Hardie et al., 1994; Hrabak et al., 2003). Several research results have suggested the roles of SNF1 in plants. A plant homologue of SNF1, SNF1-related protein kinase-1 (SnRK1) has been identified in various plants (Crawford et al., 2001; Halford et al., 2003) and showed high sequence identity with SNF1 in yeast. Expression of an antisense SnRK1 sequence in the tubers of transgenic potato plants resulted in loss of sucrose induction of sucrose synthetase gene (Purcell et al., 1998). SnRK1 is also required for α-amylase gene expression in cultured wheat embryos and expression of α-Amy1 and α-Amy2 was upregulated by sugar starvation (Laurie et al., 2003). Another result showed that SnRK1 is involved in sucrose-specific sensing, leading to redox modulation of ADP-glucose pyrophosphorylase (AGPase) in potato tubes (Tiessen et al., 2003).
1.1.2.3.2 *Crosstalk with phytohormones*

Mutants are pivotal tools to identification of genes required for a specific biological process and structure-function analysis. To understand the molecular mechanisms involved in the sugar sensing and signaling networks it has been necessary to isolate sugar response mutants. This can be done either by developmental phenotype or by reporter gene expression. Characterization of these mutants has implicated crosstalk between different signaling systems, especially those of sugars, ethylene, and ABA (Rolland et al., 2002; Leon and Sheen, 2003).

*Sugar and ethylene*

Phenotypic and genetic analysis of the *glucose-insensitive, gin1* mutant revealed the interaction between sugar and ethylene-mediated signaling pathways (Zhou et al., 1998). The phenotype of *gin1* was phenocopied by treatment with the ethylene precursor ACC (1-aminocyclopropane-1-carboxylate) and 6% glucose to the wild-type. The double mutant of *gin1* and ethylene insensitive *ein2* provides evidence for glucose-dependent developmental arrest could be overcome by ethylene (Zhou et al., 1998). Two *Arabidopsis* mutants *sis1* (Gibson et al., 2001) and *gin4* (Rolland et al., 2002) are allelic to the ethylene constitutive response mutant, *ctr1* (Kieber et al., 1993). Several *ethylene-insensitive* mutants *etr1, ein2, ein3* and *ein6* showed oversensitivity to glucose (Wang et al., 2002). This antagonistic connection is strongly supported by the discovery that the ethylene overproduction mutant *eto1* and ethylene constitutive signaling mutants *ctr1* are glucose insensitive. Results of the analysis of sugar signaling mutants have suggested that
ethylene acts as an antagonist of the glucose response. Double mutant analysis suggested that GIN1 acts downstream of the ethylene receptor, ETR1 (Zhou et al., 1998). EIN3 (ethylene-insensitive3) and EIL1 (Arabidopsis EIN3-like protein) protein stability is enhanced by ethylene (Guo and Ecker, 2003) but reduced by glucose (Yanagisawa et al., 2003).

**Sugar and ABA**

The glucose signaling pathway is also connected with the ABA response pathway. Several independent groups have isolated mutants with abnormal responses to sugars. Interestingly, a large number of mutants turned out to be ABA responsive or biosynthetic mutants. For instance, gin6, isi3, sis5, and sun6 are allelic to the ABA-responsive mutant *abi4*. The *abi4* mutant was originally selected by its ability to germinate in the presence of high levels of exogenous ABA, which normally inhibits wild-type germination and seedling development (Finkelstein et al., 1998). The predicted protein product of ABI4 contains a conserved DNA binding domain, the APETALA 2 (AP2) that plays a major role during seed development and germination together with two other loci, ABI3 and ABI5 (Finkelstein et al., 1998). Similarly, the glucose-insensitive mutant *gin5* is allelic to the ABA biosynthesis mutant *aba3* (Arenas-Huertero et al., 2000). In addition to *abi4*, it was found that the *abi5* mutant also displayed a glucose-insensitive phenotype (Arenas-Huertero et al., 2000; Laby et al., 2000). Overexpression of ABI5 showed hypersensitivity to sugars and ABA (Brocard et al., 2002). The ABI5 gene encodes a transcription factor that belongs to a large basic leucine zipper (bZIP) domain family (Finkelstein and Lynch, 2000b). Together these results showed that plant growth and
development are regulated by the crosstalk between ABA and sugar signaling. Although ABA and ethylene were previously defined as stress hormones, they interact with sugar signals under the condition used for sugar signaling mutant screens. The double mutants of gin1 x etr1 and gin1 x ein2 display a glucose insensitive phenotype, suggesting that GIN1/ABA2 is downstream of ethylene response. Thus, ethylene seems to affect glucose signaling through ABA and to promote germination and seedling development. Based on the elevated ABA level found in the ein2 mutant, it is likely that ethylene signaling partially represses the biosynthesis of ABA (Beaudoin et al., 2000; Ghassemian et al., 2000).

**1.2 NITROGEN SENSING**

Nitrogen is one of most important macronutrients that influences plant growth and development. In higher plants, nitrogen is acquired from the environment as nitrate (NO$_3^-$), ammonium (NH$_4^+$), or in the case of plant hosts for nitrogen-fixing bacteria, acquired fixed nitrogen from bacterial endosymbionts. Among these nitrogen-containing compounds, nitrate is the major source of nitrogen for plant growth in soils. Nitrate affects a variety of physiological and developmental processes in plants, including root branching, leaf expansion, and the allocation of resources between shoot and root growth (Forde, 2002).
The nitrate-inducible *Arabidopsis* gene ANR1 encodes a MADS box transcription factor that acts as a positive regulator of lateral root proliferation (Zhang and Forde, 1998). DNA microarray analyses have also been used to determine the effects of inorganic nitrogen sources on gene expression globally in plants. In *Arabidopsis*, those genes encoding nitrate reductase, the nitrate transporter NRT1, and glutamate synthase were induced by nitrate. Regulatory proteins such as an MYB transcription factor, a calcium antiporter, and putative protein kinases and metabolic enzymes, including transaldolase and transketolase of the nonoxidative pentose pathway, malate dehydrogenase, asparagine synthetase, and histidine decarboxylase were also induced (Wang et al., 2000). Another microarray analyses in tomato (Wang et al., 2001) and *Arabidopsis* (Wang et al., 2003) also showed novel nitrate-responsive genes encoding metabolic and potential regulatory proteins were found. These genes encoded enzymes in glycolysis (glucose-6-phosphate isomerase and phosphoglycerate mutase), in trehalose-6-P metabolism (trehalose-6-P synthase and trehalose-6-P phosphatase), in iron transport/metabolism (nicotianamine synthase), and in sulfate uptake/reduction. The cluster analysis in our microarray data also revealed that about 8% of the glucose-responsive genes showed altered expression when nitrogen was present, suggesting an interaction between sugar and nitrogen (Price et al., 2004).

**1.2.1 Possible nitrogen sensing mechanism in plants**

Carbon and nitrogen are the two most important elements required for plant growth and development. To cope with fluctuation in carbon and/or nitrogen sources during the life cycles, plants need sophisticated coordination systems for regulating
carbon and nitrogen metabolism. Several nitrogen assimilatory genes such as those encoding the enzyme nitrate reductase (NR), glutamine synthetase (GS), asparagine synthetase (AS), PII-like homolog (GLB1), and putative glutamate receptor (AtGLR1.1) are modulated by carbon and organic nitrogen metabolites (Cheng et al., 1992; Lam et al., 1994; Hsieh et al., 1998; Oliveira and Coruzzi, 1999; Kang and Turano, 2003). PII is a nuclear-encoded chloroplast protein allosteric effector in E. coli and other bacteria that indirectly regulates glutamine synthetase at the transcriptional and posttranslational levels in response to nitrogen availability. Analyses of transgenic plants supported the notion that the plant PII-like protein may serve as part of a complex signal transduction network involved in perceiving the status of carbon and organic nitrogen (Coruzzi and Zhou, 2001). AtGLR1.1-deficient Arabidopsis (antiAtGLR1.1) lines exhibited a conditional phenotype that was sensitive to the C/N ratio. In the presence of sucrose antiAtGLR1.1 seeds did not germinate, but germination was restored by coincubation with NO₃⁻, but not NH₄⁺ (Kang and Turano, 2003).

1.2.2 ACT domain-containing protein kinase genes

In bacteria, ACT domains (aspartokinase, chorismate mutase and prephenate dehydrogenases (TyrA)) have been shown to serve as amino acid-binding sites for feedback regulation of metabolic enzymes involving in nitrogen metabolism (Chipman and Shaanan, 2001). ACT domains are linked to a wide range of metabolic enzymes that are regulated by amino acid concentration. The uncharacterized proteins that contain single or multiple copies of the ACT domain may be novel sensors or regulators that bind specific ligands, primarily amino acids (Aravind and Koonin, 1999). Pairs of ACT
domains bind specifically to a particular amino acid leading to regulation of the linked enzyme. The example of the ACT domain in enzymes is 3-phosphoglycerate dehydrogenase (3PGDH), aspartokinase, and acetolactate synthase, which are examples of allosteric regulation by the end products of the respective pathways (Chipman and Shaanan, 2001). ACT domain in several enzymes involved in the metabolism of different amino acids and in the purine metabolism.

The ACT domain is found in GlnD and SpoT/RelA. The *E. coli* sensor protein GlnD acts as the primary nitrogen sensor in the nitrogen regulation (Ntr) system (Merrick and Edwards, 1995). In addition, RelA and SpoT proteins are involved in the synthesis of guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp), compounds that play a central role in repression of tRNA and rRNA synthesis during amino acid starvation (stringent response) (Martinez-Costa et al., 1998). It is likely that the catalytic domains of these enzymes are regulated in response to yet unidentified ligands bound by their ACT domains.

In *Arabidopsis*, there are at least eight ACT domain repeat (ACR protein family) containing proteins (Hsieh and Goodman, 2002). Northern-blot analysis indicated that the ACT domain repeat gene family has a distinct expression pattern in various organs and seedlings grown in tissue culture with different chemicals. The expression of the ACR gene family is differentially regulated by ABA, BA, salt stress, cold stress, and light/dark treatment. The amino acid sequences of *Arabidopsis* ACR proteins are similar in the ACT domains to the bacterial sensor protein GlnD (Hsieh and Goodman, 2002). Two ACT domain-containing protein kinase genes (*ACTPKs*), which are highly regulated by glucose, were discovered by recent DNA microarray analysis (Price et al., 2004). In these
genes, the ACT domain is linked to protein kinase and the putative kinase activity could be regulated by glucose, amino acids, or both. Therefore, the ACTPK may function as a sensor protein in C/N sensing in plants.

### 1.3 CURRENT PROBLEMS AND POSSIBLE SOLUTIONS

To date, various molecular, phenotypic, and genetic analyses have unraveled mechanisms of sugar sensing and signaling pathway as well as various gene expression changes involve metabolism, growth and development, stress response, and senescence. Circumstantial evidence has suggested that several intermediates such as protein phosphatase, kinases, and Ca^{2+} act as second messengers in sugar signaling. In addition, several transcription factors have been found to mediate sugar responsive gene expression (Lu et al., 2002; Cakir et al., 2003; Sun et al., 2003).

Extensive mutant screens of sugar sensing and signaling have suggested sugar, ethylene, and ABA interplay in seed germination and in other developmental and physiological processes. In addition, analysis of mutants and the corresponding genes have identified regulatory components in sugar signaling pathway. Nonetheless, no component that directly regulates sugar signaling has been identified in these screens. Genetic analyses of sugar-signaling mutants suggested that the phytohormone ABA plays an critical role in sugar-mediated gene expression (Arenas-Huertero et al., 2000; Huijser et al., 2000; Laby et al., 2000; Rook et al., 2001). These results indicated that ABA
accumulation induced by glucose was essential for hexokinase-mediated sugar response. Although the analysis of sugar-insensitive mutants has provided some insights into sugar sensing and signaling in plants, the components identified so far are mostly involved in either ABA biosynthesis or ABA perception, suggesting a stress condition has been created during the mutant screen. Furthermore, several questions from these studies still remain to be answered: 1) what’s the role of ABA in sugar signaling; 2) how sugar signaling and ABA signaling crosstalk; and 3) whether ABA-independent sugar-signaling pathways exist. Thus, a different mutant selection scheme is needed to avoid obtaining the same ABA related mutants and to increase the chance to identify additional components in plant sugar signal transduction pathways.

The traditional genetic approaches have been successfully used for searching the genes involved in signal transduction pathways in plants. In addition, the availability of the complete *Arabidopsis* genome sequence has made high-throughput approaches possible in unraveling sugar signal transduction mechanisms. The comparison of global gene expression profiles between sugar-treated plant and wild-type plant or sugar mutant and wild-type plant will facilitate the genetic analysis of sugar signal transduction. In *Arabidopsis*, T-DNA insertion lines have been created that represent near saturation of the genome (Alonso et al., 2003). Combined with DNA microarray analysis, the reverse genetics approach using knockouts of sugar response genes encoding enzymes of undefined function can lead to the discovery of gene functions and reveal new pathways of plant metabolism (Thorneycroft et al., 2001). For example, comparison of gene expression profiles between wild-type and mutant *pho3*, which accumulates high levels of sucrose (Zakhleniuk et al., 2001), revealed a striking increase in the expression of
genes associated with the plastid glucose 6-phosphate/phosphate translocator, starch synthesis, and very large increases were observed in the expression of transcription factors and enzymes involved in anthocyanin biosynthesis (Lloyd and Zakhleniuk, 2004). These findings suggest that gene expression analysis using DNA microarray in sugar mutants can provide a tool for investigating the genomic response to sugar in plants.

1.4 STRATEGIES TO STUDYING SUGAR SENSING AND SIGNALING IN ARABIDOPSIS

1.4.1 Dissection of the glucose signal transduction pathway in Arabidopsis thaliana using an improved genetic screen.

A combination of genetic, molecular, biochemical, and physiological methods has been used to identify genes involved in perception and transduction of sugar signals. Several groups have isolated mutants in *Arabidopsis* based on reversible inhibition of seedling development under high exogenous sugar concentrations (Leon-Kloosterziel et al., 1996; Zhou et al., 1998; Pego et al., 2000; Gibson et al., 2001). In addition, mutants with altered gene expression in response to sugar were also isolated by *in vivo* reporter-based screens in transgenic *Arabidopsis* plants harboring: a) the light- and sugar-regulated plastocyanin promoter fused to the luciferase coding sequence (Dijkwel et al., 1997), b) sugar responding *Pat* (*B33*)-*iudA* construct (Martin et al., 1997), c) sugar responding *Atβ*-Amy transcripts encoding a β-amylase gene (Mita et al., 1997a; Mita et
al., 1997b), and d) sugar inducible ApL3 gene (large subunit of ADP-glucose pyrophosphorylase subunit involved in starch biosynthesis) (Sokolov et al., 1998) fused to a negative selection marker (ApL3::P450) gene (Rook et al., 2001). Furthermore, a transgenic line containing ApL3 promoter linked to firefly luciferase reporter was used for mutant isolation on medium containing 1% (w/v) sucrose. Several high sugar-response (hsr) mutants isolated from this screen showed altered responses to low levels of sucrose and glucose. These mutants did not display ABA or ethylene responses (Baier et al., 2004). Interestingly, the previous screens using ApL3 gene also found two mutants, isi1 and isi2, that did not exhibit an ABA-related phenotype (Rook et al., 2001). On the basis of identities of mutants isolated from above screens, mutant screens based on phenotype may accompany stress conditions due to the fact that high sugar levels are required to arrest plant development to differentiate mutant from WT. By contrast, in vivo reporter-based screen systems may avoid these stress conditions by decreasing sugar levels on screening conditions to the physiological relevant levels in plants by the application of sugar-sensitive marker gene fused with in vivo reporter gene. On the grounds of the hypothesis that sugar signals can be transmitted through unique signaling pathways that are not involved in other hormone or stress related signaling pathways, I have developed a genetic screen using marker genes that are sensitive to low concentrations of glucose. In this research, Asparagine Synthetase1 (ASN1) was used as a sugar marker to select trans mutations that can affect the expression of ASN1 gene. Fusion constructs between ASN1 promoter and reporter, firefly luciferase (LUC) or green fluorescent protein (GFP) were transformed into Arabidopsis. The resulting T3 homozygous plants were mutagenized to select the second-site mutation that could affect
the expression of the *ASN1* in response to sugar. Cloning and characterization of these mutants could help to elucidate the mechanism of glucose signaling in higher plants.

### 1.4.2 Investigate the role of the ACT domain-containing protein kinase family in C/N sensing

In bacteria, ACT domains have been shown to serve as amino acid-binding sites for feedback regulation of metabolic enzymes involving in nitrogen metabolism. I have found that the transcription of two ACT domain-containing protein kinases was highly regulated by glucose. I found three putative ACT domain-containing protein kinase genes (*ACTPK*) from databases. I propose that the *ACTPK* may function as sensor protein in C/N sensing in plants.

**Therefore, the specific objectives were:**

1. To characterize a sugar-sensitive marker gene *ASN1* in *Arabidopsis*.
2. To construct a forward genetic screen system to be used in the dissection of ABA-independent sugar signal transduction pathway.
3. To identify mutants with altered glucose response.
4. To determine the role of ACT domain-containing protein kinase (*ACTPK*) genes in C/N sensing.
CHAPTER 2

REGULATION OF ASPARAGINE SYNTHETASE GENE (ASN1) EXPRESSION

2.1 ABSTRACT

Recent studies have shown that abscisic acid (ABA) and ethylene interact closely with the glucose signaling pathway in the control of Arabidopsis seedling development. Although these results indicated that ABA might act as a second messenger for sugar signal transduction, they might not reflect a physiological role of sugar signaling for normal plant growth and development. Therefore, it is important to use a refined mutant selection scheme to identify mutants that are not directly related to ABA. To identify a unique sugar-signaling pathway, I attempted to select trans mutations that can affect the expression of a marker gene. Since I wanted to identify mutations that could reflect different aspects of sugar signaling than known ABA-related mutants, it was crucial to select a good marker that would respond sensitively and specifically to low glucose concentration. I found that Asparagine Synthetase1 (ASN1) was completely repressed by
low levels of exogenous glucose (0.1%, w/v) in 2 h but was derepressed upon the removal of glucose. In addition, suppression of $ASN1$ by glucose could not be rescued by the addition of organic nitrogen source, asparagine (Asn). While both glucose and mannose, efficient substrates of HXK, could repress $ASN1$ expression, a non-substrate of HXK, 3-$O$-methyl-glucose, and a non-transportable sugar, L-glucose, did not cause any repression on $ASN1$. These results suggested that $ASN1$ repression required sugar transport, phosphorylation, and HXK. By treating with cycloheximide (CHX), a general protein synthesis inhibitor, I also found that the glucose repression of $ASN1$ does not require de novo protein synthesis. The derepression of $ASN1$ gene expression in the absence of sugar in the medium was inhibited by serine/threonine protein kinase inhibitor, K-252a, but not by the protein phosphatase inhibitor okadaic acid, suggesting a requirement of protein phosphorylation in $ASN1$ de-repression. I have further tested whether this glucose repression was independent of ABA. Results by molecular analysis showed that the repression of $ASN1$ occurred even in the presence of fluridone, an ABA biosynthesis inhibitor and surprisingly, ABA could enhance $ASN1$ expression. Nevertheless, glucose induced $ASN1$ repression persisted in the presence of exogenous ABA. This result indicated that $ASN1$ repression by glucose was not mediated through ABA under the conditions used in my experiments. In the current study, I addressed the question of how plants sense sugars, and how sugar triggers $ASN1$ repression in Arabidopsis.
2.1 INTRODUCTION

In an effort to understand the complex sugar sensing and signaling system in plants, various mutants have been isolated and characterized by different screening strategies. To screen for sugar-unresponsive mutants, I used a reporter-based screening procedure in which promoter of sugar-repressible genes were linked to reporter genes. Among several sugar repressible genes, I selected *Asparagine Synthetase1 (ASN1)* as a sugar-repressible marker gene.

In plants, asparagine plays an important role in nitrogen storage and transport because it contains a high N/C ratio and it has relatively good solubility, stability, and mobility in the cells. There are three proposed routes for the synthesis of asparagine (Sieciechowicz et al., 1988). Among these routes, the glutamine-dependent pathway has been accepted as a major route in plants. This pathway utilizes asparagine synthetase (AS; the enzyme catalyzing the biosynthesis of asparagines) to catalyze an ATP-dependent reaction that can transfer the amide group from glutamine to aspartate to produce asparagine, glutamate, AMP, and PPi. Although asparagine was the first amino acid discovered in plants (1896), the characterization of plant AS enzymes were delayed due to enzymatic instability in partially purified extracts and the problem of purification (Romagni and Dayan, 2000). However, the advance of molecular techniques has enabled the cloning of two *ASN* cDNAs from pea by using human *ASN* cDNA as a heterologous probe (Tsai and Coruzzi, 1990). Additional *ASN* genes have been subsequently isolated.
from legume, nonlegume, and monocot plants. In *Arabidopsis*, three *ASN* genes (*ASN1*, *ASN2*, and *ASN3*) has been cloned (Lam et al., 1994; Lam et al., 1998). Among the three *ASN* genes, *ASN1* and *ASN2* were reciprocally regulated by light and metabolites. While *ASN1* was repressed, *ASN2* was induced by sucrose in time course experiments. Sucrose repression of *ASN1* is partially relieved after 48 h in the presence of exogenous glutamine, glutamate, or asparagine. In contrast, *ASN2* was repressed by these amino acids. With light exposure, *ASN1* was repressed, while *ASN2* was induced significantly only with ammonium in medium (Lam et al., 1998; Wong et al., 2004). Recent research on transgenic *Arabidopsis* constitutively overexpressing *ASN1* (35S-*ASN1*) suggested that *ASN1* play a major role in nitrogen assimilation, regulating nitrogen transport, and storage during seed development (Lam et al., 2003). In addition, several dark-inducible genes (*DIN*) were isolated from *Arabidopsis* using differential display (Fujiki et al., 2000). Among many genes, *DIN6* gene turned out to be identical to previously known *ASN1*. The results suggested that enhanced expression of *DIN* genes triggered by sugar starvation occurred in leaf cells when the photosynthesis was hindered (Fujiki et al., 2000; Fujiki et al., 2001). In this research, *ASN1* gene was examined in various conditions to use as a sugar-sensitive marker gene for construction of a forward genetic screen system in *Arabidopsis*. 
2.3 MATERIALS AND METHODS

Plant Material and Growth Condition

Wild-type and transgenic Arabidopsis plants were the Columbia-0 ecotype unless otherwise specified. Seed batches were grown simultaneously, harvested, and stored for at least a month before using them in the experiments. For in vitro growth, a liquid culture system (Cheng et al., 1992) was employed in the experiments for gene expression analysis. Seeds were surface-sterilized in 95% (v/v) ethanol for 1 min followed by 50% (v/v) commercial bleach (Clorox) for 15-20 min, and rinsed five times with sterile water. After 3 d stratification period at 4°C in the dark, approximately 1,000 seeds were transferred into 125 ml Erlenmeyer flasks containing 20 ml 1x basal Murashige and Skoog media (MS) (Murashige and Skoog, 1962) and left at 4°C for another 4 d complete stratification. Plants were grown on a rotary shaker (New Brunswick Scientific, Edison, NJ) with constant agitation at 128 rpm under constant light (90 μmol m⁻²s⁻¹) at 22°C for 6 d. Seedlings were washed three times with sugar and nitrogen free MS medium then transferred to the dark conditions for 1 d in a sugar-free medium to deplete endogenous sugars. Plants were collected for RNA extraction at the end of treatments.

Treatments with chemical antagonists

Cycloheximide was obtained from Sigma-Aldrich (St. Louis, MO) and dissolved in distilled water at 10mM. Okadaic acid and K-252a were purchased from A.G. Scientific, Inc. (San Diego, CA) and dissolved in dimethyl sulfoxide (DMSO) at 1mM to yield stock solution. After the addition of the selected reagents, the flasks were returned
to the original culture condition. For controls, seedlings were treated with equivalent amounts of distilled water or DMSO; these treatments did not affect transcript levels.

**RNA isolation, RT-PCR, and Northern blot analysis**

Ground plant material was frozen by liquid nitrogen and RNA was extracted by using either RNeasy® Plant Mini Kit (Qiagen, Hilden, Germany) according to the modified manufacturer’s protocol or our laboratory protocol (Xiao et al., 2000). All experiments were repeated at least twice with RT-PCR and Northern blot analysis. The cDNA samples and RNA amount were standardized with analysis of spectrophotometer and ethidium bromide gel. Total RNA (0.5 µg) was reverse-transcribed using the Takara Bio RT-PCR kit following a modified version of manufacturer’s protocol (Takara One-Step RNA PCR kit, Madison, WI). PCR was performed in a 25 µL reaction mixture containing 0.5 µg of total RNA, 2.5 µl 10x one step RNA PCR buffer, 5 µl 25 mM MgCl2, 2.5 µl 10m mol dNTPs, 0.5 µl RNase inhibitor (40U µl⁻¹), 0.5 µl AMV RTaseXL (5U µl⁻¹), 50 ng of each gene-specific primers, *AtASN1* (GenBank accession no. L29083), forward, 5’-TTGCTCACTTGATACGAG-3’; reverse, 5’-ATTGCTTAGCCGCTTTA-3’; *AtHXK1* (GenBank accession no. U28214), forward, 5’-CAGCAGCTTGTTGGTTT-3’, reverse, 5’-GCTTCTTCGTCAACCTG-3’; *AtRab18* (GenBank accession no. X68042), sense: 5’-ACTGACGAGTACGGAAA-3’, reverse, 5’-TGATCCTTGTCCATCATC-3’. The PCR primers used for detection of *AtASN1*, *AtHXK1*, and *AtRab18* yielded cDNA products of 468 bp (*AtASN1*), 500 bp (*AtHXK1*), and 578 bp (*AtRab18*). The conditions for PCR were one cycle of denaturing at 94 °C for 2 min, followed by 25 cycles of at 94 °C for 30 sec, 50 °C for 30 sec, 72 °C for 1 min 30 sec. Three microliter of PCR products were
loaded onto 1% agarose gels, electrophoresed and stained with 0.5 mg/ml ethidium bromide.

For Northern blot analysis, 5 µg of each total RNA sample was loaded on 1.2% (w/v) agarose gels and subsequently blotted onto a nylon membrane (ICN, Irvine, CA), using standard procedures (Sambrook et al., 1989). To fix RNA to the blot, blot was exposed to UV using UV crosslinker (Invitrogen, Carlsbad, CA) with 0.120 J and subsequently baked at 80°C for 1h. Radiolabeled DNA probes were prepared by PCR reactions with 20 µl mixture containing 1x labeling buffer, 0.2 mM deoxynucleotide triphosphate (without ATP), 0.25 units of Taq polymerase (New England Biolab, Beverly, MA), 0.05 µg primers and 0.1 µg template DNA, and 1 µCi ³²P-α-ATP. Thirty-six cycles of PCR amplification were carried out at 94°C for 30 sec (denaturation), 50-54°C for 45 sec (annealing) and 72°C for 60 sec (synthesis). For Northern blot analysis, a cDNA clone (EST) of Arabidopsis ASN1 (GenBank accession no. 135B13T7) was obtained from the Arabidopsis Biological Resource Center (ABRC) at The Ohio State University. This clone was used as a template DNA for radioisotope probe preparation. The probe was labeled with radioisotope (³²P-α-ATP) using the T7 and M13 primer. The radiolabeled probe was separated from the unincorporated dNTPs by chromatography through a spin column of Sephadex G-50. The purified radiolabeled cDNA were used for hybridization with denaturation by heating for 5 minutes at 100°C and chilling the probe rapidly in ice water. Radiolabeled cDNA activity of 1.5 x 10⁶ cpm ml⁻¹ was used for each membrane. That membrane was hybridized for 12-16 hours in a rotating hybridization oven (Robbins Scientific, Sunnyvale, CA) and washed in 500 ml of 2x SSC containing 1% (w/v) SDS for 15 min at room temperature then washed 3 times with 0.5x
SSC containing 1% (w/v) SDS for 30 min at 60°C, and then subjected to autoradiography.

2.4 RESULTS

2.4.1 High concentrations of glucose causes ABA accumulation and RAB18 induction

In an attempt to characterize metabolic regulation of *ASN1*, I tested gene expression by Northern blotting and RT-PCR analyses. It is generally assumed that high levels of exogenous glucose cause ABA accumulation, and that results in a delay of germination and an inhibition of early seedling development. I test this hypothesis using the ABA sensitive marker *RAB18* (responsive to ABA) (Welin et al., 1994; Nylander et al., 2001) was used and found to exhibit accumulation patterns in response to increasing on glucose concentration (Fig. 2.1). Using this ABA marker, I also found that the condition that causes *ASN1* repression is not sufficient to induce *RAB18* (Fig. 2.12).

2.4.2 Cross-regulation of *ASN1* by light and glucose

Although I knew *ASN1* mRNA in dark-grown plants was strongly repressed by the presence of exogenous sucrose from the previous study (Lam et al., 1994), the glucose effect on *ASN1* mRNA was not clear. In order to determine the effects of glucose or light on *ASN1* expression, I conducted Northern-blot and RT-PCR analyses. Plants were grown 6 days under continuous illumination supplemented with 3% (w/v) glucose containing Murashige and Skoog (MS) medium then transferred to a sugar-free medium.
with dark conditions for 1 day, and then collected from the medium after varying periods of time. Three percent glucose strongly suppressed $ASN1$ expression in 3 h under light (Fig. 2.2). A previous study showed that light had negative effect on $ASN1$ transcript levels in *Arabidopsis* (Lam et al., 1994). To further test the effect of light and dark on $ASN1$ gene regulation, plants were grown under 4 days continuous light or dark in different concentrations of glucose in the medium. Transcripts of $ASN1$ gene were highly expressed at dark-treated and sugar-starved plants (at time 0). In addition, as glucose concentration increased, the $ASN1$ transcripts were decreased (Fig. 2.3). Light also repressed $ASN1$ expression level, but transcripts were not abolished completely. Application of 3% (w/v) glucose eliminated $ASN1$ transcripts in both conditions.

To examine the temporal and dose effects of sugar on $ASN1$ gene regulation, seedlings were transferred to a sugar-free medium in dark conditions for 24 h to maintain the highest transcript levels and then seedlings were returned to glucose-containing medium. In the presence of exogenous 3% (w/v) glucose, $ASN1$ expression was diminished in 1 h and was completely abolished in 3 h (Fig. 2.4). Furthermore, as little as 0.1% (w/v) glucose was sufficient to eliminate the accumulation of $ASN1$ transcripts (Fig. 2.4). Similar to glucose, light also repressed $ASN1$ expression. However, while it only took 3 h to completely repress $ASN1$ with glucose, it took 24 h with light (Fig. 2.5). To examine the expression of $ASN1$ gene during sugar starvation, seedlings were transferred to 3% (w/v) glucose in dark conditions for 24 h and then returned to a sugar-free medium (Fig. 2.6). While $ASN1$ was completely repressed by low levels of exogenous glucose in 3 h, it was derepressed by removing exogenous glucose from the culture medium took 24 h (Fig. 2.6).
Figure 2.1. Higher concentrations of glucose cause induction of ABA marker gene RAB18 presumably due to ABA accumulation.

RT-PCR analysis was used to determine the steady state mRNA level of Rab18. The WT (Col-0) plants were grown in MS liquid medium with various concentrations of glucose in continuous light or dark for 4 days before tissue collection and RNA extraction. Two microliters of RT-PCR product was loaded in each lane. Equal loading is indicated by uniform intensity of rRNA bands stained by ethidium bromide.
Figure 2.2. Kinetics of light and glucose repression of *ASN1*.

RNA gel blot analysis was used to determine the steady state mRNA level of *ASN1*. The WT (Col-0) plants were grown in MS liquid medium with 3% glucose in continuous light for 6 days. Plants were then washed 3 times with sugar and nitrogen free MS medium and dark adapted for 24 h to deplete internal sugar and nitrogen. Plants were then treated with 3% glucose in the light for various amount of time before tissue collection and RNA extraction. Five micrograms of total RNA was loaded in each lane. Equal loading is indicated by uniform intensity of rRNA bands stained by ethidium bromide. **MS 3 h:** RNA sample collected from identically prepared plants except that they were incubated in sugar-free MS in the dark for 3 h in the last step. **0:** RNA sample collected from plants after the dark adaptation. **Continuous light:** RNA collected from plants grown in MS with 3% glucose in continuous light for 8 days.
Figure 2.3. Light and glucose affects *ASN1* mRNA levels.

Northern blot analysis was used to determine the steady state mRNA level of *ASN1*. The WT (Col-0) plants were grown in MS liquid medium with various concentrations of glucose in continuous light or dark for 4 days before tissue collection and RNA extraction. Five micrograms of total RNA was loaded in each lane. Equal loading is indicated by uniform intensity of rRNA bands stained by ethidium bromide.
Figure 2.4. Kinetics of glucose repression of \textit{ASN1} with time and dose.

RNA gel blot analysis was used to determine the steady state mRNA level of \textit{ASN1}. The WT (Col-0) plants were grown in MS liquid medium with 3% glucose in continuous light for 6 days. Plants were then washed 3 times with sugar and nitrogen free MS medium and dark adapted for 24 h to deplete internal sugar and nitrogen. Plants were then treated either with 3% glucose in the dark for various amount of time (time-course experiment) or with various concentrations of glucose in the dark (glucose dose test) before tissue collection and RNA extraction. Five micrograms of total RNA was loaded in each lane. Equal loading is indicated by uniform intensity of rRNA bands stained by ethidium bromide. \textbf{MS 3 h}: RNA sample collected from identically prepared plants except that they were incubated in sugar-free MS in the dark for 3 h in the last step. \textbf{0}: RNA sample collected from plants after the dark adaptation.
Figure 2.5. Kinetics of light repression of *ASN1*.

RT-PCR analysis was used to determine the relative mRNA expression of *ASN1*. The WT (Col-0) plants were grown in MS liquid medium with 3% glucose in continuous light for 6 days. Plants were then washed 3 times with sugar and nitrogen free MS medium and dark adapted for 24 h to deplete internal sugar and nitrogen. Plants were then treated with sugar-free medium for various amount of time in the light before tissue collection and RNA extraction. Two microliters of RT-PCR product was loaded in each lane. Equal loading is indicated by uniform intensity of rRNA bands stained by ethidium bromide. 0: RNA sample collected from plants after the dark adaptation.
Figure 2.6. Kinetics of glucose derepression of \textit{ASN1}.

RT-PCR analysis was used to determine the relative mRNA expression of \textit{ASN1}. The WT (Col-0) plants were grown in MS liquid medium with 3% glucose in continuous light for 6 days. Plants were then dark-adapted for 24 h. Before transferring into sugar-free medium for various amount of time in the dark before tissue collection and RNA extraction. Two microliters of RT-PCR product was loaded in each lane. Equal loading is indicated by uniform intensity of rRNA bands stained by ethidium bromide. 0: RNA sample collected from plants after the dark adaptation.
2.4.3 Cross-regulation of ASNI by nitrogen and glucose

To test the effects of amino acid supplementation on ASNI transcription, Arabidopsis seedlings were grown in MS medium, which contained 60 mM inorganic nitrogen sources, and then treated with glucose or Asn. Asn was chosen as an organic nitrogen source since it has high nitrogen to carbon ratio compared to the other amide amino acids. Our preliminary data showed that ASNI gene induction by inorganic nitrogen such as nitrate and ammonium were overridden by low levels of glucose (0.1%, w/v). While sucrose (3%, w/v) could not suppress induction of ASNI by Asn (0.4 mM) (Lam et al., 1994), our results showed lower levels of glucose (0.5%, w/v) suppressed ASNI transcripts in the presence of Asn (0.4mM) (Fig. 2.7).

2.4.4 The role of hexokinase

AtHXK1 plays an important role in sugar signaling (Jang et al., 1997) and AtHXK expression is induced by sugar (Price et al., 2004). To investigate whether ASNI repression by glucose requires the expression of HXK, I first examined HXK1 steady state mRNA level in a time course experiment. Results showed that the timing of HXK1 induction coincided with ASNI repression caused by glucose (Fig. 2.8). To further investigate the involvement of HXK in glucose-induced ASNI repression, I tested the effects of different sugars and glucose analogs (Fig. 2.9). Glucose and mannose, both efficient substrates of HXK, repressed ASNI expression. Although sucrose is not a substrate of HXK, it can be hydrolyzed into glucose and fructose, which also caused a strong repression of ASNI. In contrast, an inefficient substrate of HXK 3-O-methyl-glucose and a non-transportable sugar, L-glucose, did not cause any repression on ASNI.
Figure 2.7. The effects of nitrogen and glucose on the expression of *ASN1*. RT-PCR analysis was used to determine the relative level of steady state mRNA levels. The WT (Col-0) plants were grown in MS liquid medium with 3% glucose in continuous light for 6 days. Plants were then washed 3 times with sugar and nitrogen free MS medium and dark adapted for 24 h to deplete internal sugar and nitrogen. Plants were then treated with various amounts of glucose and/or Asparagine (Asn) in the dark before tissue collection and RNA extraction. Two microliters of RT-PCR product was loaded in each lane. Equal loading is indicated by uniform intensity of rRNA bands stained by ethidium bromide.
Mannitol did not cause \textit{ASN1} repression suggesting the effect of glucose is not a result of osmotic change. These results suggest that \textit{ASN1} repression involve sugar transport and phosphorylation and \textit{ASN1} repression by sugars required HXK. To further verify the effect of HXK in \textit{ASN1} expression, I examined the transcript level of \textit{ASN1} in WT and plants with altered HXK levels (Fig. 2.10). In all genotypes, addition of exogenous glucose reduced the expression of \textit{ASN1}. In the absence of glucose, \textit{ASN1} was expressed at a lower level in 35S:AtHXK1 plants (\textit{HXK1} overexpressor) than in the wild-type (Bensheim, BE), presumably due to the repression by endogenous glucose. This conclusion is supported by the fact that 35S:AtHXK1 is known to be hypersensitive to sugar (Zhou et al., 1998; Xiao et al., 2000). The level of \textit{ASN1} expression in gin2, an \textit{AtHXK1} knockout mutant, was similar to that in the wild-type (Landsberg \textit{erecta}, Ler), which can be ascribed to the redundant function of other hexokinases or glucokinases to \textit{AtHXK1} (Jang et al., 1997).

\textbf{2.4.5 The role of ABA in glucose repression of \textit{ASN1}}

Recent studies showed that cellular ABA concentration was induced by high concentration of exogenous glucose (Garcia-Rubio et al., 1997; Finkelstein and Lynch, 2000a). Although I have shown that low level of glucose (0.1%) can repress \textit{ASN1}, it was still not clear whether this repression could be modulated by ABA, i.e. whether the repression could be mediated indirectly through the accumulation of ABA. Results showed that the repression of \textit{ASN1} occurred even in the presence of 100 µM fluridone (1-methyl-3-phenyl-5-(3-[trifluoromethyl]phenyl)-4-(1H)-pyridinone), an ABA biosynthesis inhibitor (Moore and Smith, 1984; Lang and Palva, 1992; Moreno-Fonseca
and Covarrubias, 2001) (Fig. 2.11). This result suggests that $ASN1$ repression by glucose is not mediated through ABA. Most surprisingly, I have found that instead of causing a repression, ABA can enhance $ASN1$ expression. Nevertheless, glucose induced $ASN1$ repression persisted in the presence of exogenous ABA (Fig. 2.11). To determine the relationship between glucose and ABA, I examined whether the expression of an ABA-specific marker gene under the conditions that would cause $ASN1$ repression. I found that the conditions that cause $ASN1$ repression are not sufficient to induce $RAB18$ (Welin et al., 1994; Nylander et al., 2001) (Fig. 2.12). To further confirm these results, I used the ABA biosynthetic mutant ($aba2$) and signaling mutant ($abi4$). The glucose effects on $ASN1$ repression with these known ABA biosynthetic or signaling mutants were the same as the wild-type (Col-0) (Fig. 2.13) indicating glucose repression of $ASN1$ might not be involved in ABA signaling pathway or biosynthesis. In addition, ABA could not modulate glucose repression of $ASN1$ when both ABA and glucose were present. I cannot rule out the possibility that other ABA signaling components may affect glucose repression of $ASN1$ because other ABA mutants have not been tested yet.

### 2.4.6 The role of de novo protein synthesis in glucose repression of $ASN1$

To determine whether the repression of $ASN1$ required de novo protein synthesis, I examined $ASN1$ expression in the presence of cycloheximide (CHX), an inhibitor of protein synthesis. A time-dependent study showed that both low and high levels of CHX caused the increased $ASN1$ transcript levels within 3 h (Fig. 2.14).
2.4.7 The role of postranslational modification in glucose repression of \textit{ASN1}

In plants, protein phosphorylation and dephosphorylation events are known to regulate various enzymatic activities (Luan, 2003). To determine whether protein phosphorylation or dephosphorylation events were involved in the expression of the \textit{ASN1} gene during glucose signaling, plants were treated with 1 µM okadaic acid (OKA) or 4-µM K-252a. Okadaic acid has been known to inhibit protein phosphatase type 2A (PP2A) but not type 1 (PP1) (Bialojan and Takai, 1988) and K-252a is a general Ser/Thr protein kinase inhibitor (Schmidt et al., 1995). Results showed that K-252a strongly repressed \textit{ASN1} expression in both the absence and presence of glucose (0.1%, w/v) (Fig. 2.15) suggesting that protein phosphorylation is required for the derepression of \textit{ASN1}. 
Figure 2.8. Timing of \textit{HXK1} induction correlates with timing of \textit{ASN1} repression.

RT-PCR analysis was used to determine the relative level of steady state mRNA of \textit{ASN1}. The WT (Col-0) plants were grown in MS liquid medium with 3\% glucose in continuous light for 6 days. Plants were then washed 3 times with sugar and nitrogen free MS medium and dark adapted for 24 h to deplete internal sugar and nitrogen. Plants were then treated with MS medium with 3\% glucose for various amount of time in the dark before tissue collection and RNA extraction. Two microliters of RT-PCR product was loaded in each lane. Equal loading is indicated by uniform intensity of rRNA bands stained by ethidium bromide. 0: RNA sample collected from plants after the dark adaptation.
Figure 2.9. *ASN1* repression involves sugar transport and phosphorylation.

RNA gel blot analysis was used to determine the level of *ASN1* steady state mRNA. The WT (Col-0) plants were grown in MS liquid medium with 3% glucose in continuous light for 6 days. Plants were then washed 3 times with sugar and nitrogen free MS medium and dark adapted for 24 h to deplete internal sugar and nitrogen. Plants were then treated with 0.1% sugar or glucose analogs in the dark for 3 h before tissue collection and RNA extraction. Five micrograms of total RNA was loaded in each lane. Equal loading is indicated by uniform intensity of rRNA bands stained by ethidium bromide. **MS 3 h:** RNA sample collected from identically prepared plants except that they were incubated in sugar-free MS in the dark for 3 h in the last step.
Figure 2.10. *ASN1* repression by sugars required HXK.

RT-PCR analysis was used to determine the relative level of *ASN1* steady state mRNA. The WT (BE and Ler) plants and HXK1 mutants (*35S:AtHXK1* and *gin2*) were grown in MS liquid medium with 3% glucose in continuous light for 6 days. Plants were then washed 3 times with sugar and nitrogen free MS medium and dark adapted for 24 h to deplete internal sugar and nitrogen. Plants were then treated with/without various concentrations of glucose for 3 h in the dark before tissue collection and RNA extraction. Two microliters of RT-PCR product was loaded in each lane. Equal loading is indicated by uniform intensity of rRNA bands stained by ethidium bromide. 0: RNA sample collected from plants after the dark adaptation.
Figure 2.11. Neither exogenous ABA nor inhibitor of ABA biosynthesis could block glucose repression of *ASN1*.

RNA gel blot analysis was used to determine the level of *ASN1* steady state mRNA. The WT (Col-0) plants were grown in MS liquid medium with 3% glucose in continuous light for 6 days. Plants were then washed 3 times with sugar and nitrogen free MS medium and dark adapted for 24 h to deplete internal sugar and nitrogen. Plants were then treated with glucose, ABA, and/or Fluridone in the dark for 3 to 12 h before tissue collection and RNA extraction. Five micrograms of total RNA was loaded in each lane. Equal loading is indicated by uniform intensity of rRNA bands stained by ethidium bromide. **MS 3 h**: RNA sample collected from identically prepared plants except that they were incubated in sugar-free MS in the dark for 3 h in the last step.
Figure 2.12. Condition that causes *ASN1* repression is not sufficient to induce ABA responsive marker gene *RAB18*.

RT-PCR analysis was used to determine the relative level of *Rab18* steady state mRNA. The WT (Col-0) plants were grown in MS liquid medium with 3% glucose in continuous light for 6 days. Plants were then washed 3 times with sugar and nitrogen free MS medium and dark adapted for 24 h to deplete internal sugar and nitrogen. Plants were then treated with 3% glucose medium for various amount of time in the dark before tissue collection and RNA extraction. Two microliters of RT-PCR product was loaded in each lane. Equal loading is indicated by uniform intensity of rRNA bands stained by ethidium bromide. 0: RNA sample collected from plants after the dark adaptation.
Figure 2.13. Glucose repression of \textit{ASN1} is not blocked in mutant with defect in either ABA biosynthesis (\textit{aba2}) or ABA perception (\textit{abi4}).

RT-PCR analysis was used to determine the relative level of \textit{ASN1} steady state mRNA. The WT (Col-0) plants and ABA mutants (\textit{aba2} and \textit{abi4}) were grown in MS liquid medium with 3\% glucose in continuous light for 6 days. Plants were then washed 3 times with sugar and nitrogen free MS medium and dark adapted for 24 h to deplete internal sugar and nitrogen. Plants were then treated with MS medium with/without 3\% glucose for 3 h in the dark before tissue collection and RNA extraction. Two microliters of RT-PCR product was loaded in each lane. Equal loading is indicated by uniform intensity of rRNA bands stained by ethidium bromide.
Figure 2.14. *De novo* protein synthesis does not seem to be required for glucose repression of *ASN1*.

RNA gel blot analysis was used to determine the level of *ASN1* steady state mRNA. The WT (Col-0) plants were grown in MS liquid medium with 3% glucose in continuous light for 6 days. Plants were then washed 3 times with sugar and nitrogen free MS medium and dark adapted for 24 h to deplete internal sugar and nitrogen. Plants were then treated with glucose and CHX for various amount of time in the dark before tissue collection and RNA extraction. Five micrograms of total RNA was loaded in each lane. Equal loading is indicated by uniform intensity of rRNA bands stained by ethidium bromide. 0: RNA sample collected from plants after the dark adaptation. **MS 3 h**: RNA sample collected from identically prepared plants except that they were incubated in sugar-free MS in the dark for 3 h in the last step.
Figure 2.15. Protein phosphorylation is required for the derepression of *ASN1*.

RNA gel blot analysis was used to determine the level of *ASN1* steady state mRNA. The WT (Col-0) plants were grown in MS liquid medium with 3% glucose in continuous light for 6 days. Plants were then washed 3 times with sugar and nitrogen free MS medium and dark adapted for 24 h to deplete internal sugar and nitrogen. Plants were then treated with various concentrations of glucose and/or inhibitors for 3 h in the dark before tissue collection and RNA extraction. Five micrograms of total RNA was loaded in each lane. Equal loading is indicated by uniform intensity of rRNA bands stained by ethidium bromide.
2.5 DISCUSSION

2.5.1 Low levels of glucose repress ASN1

The accumulation of ASN1 mRNA in dark-grown plants was repressed by the presence of exogenous sucrose and light (Lam et al., 1994). A series of time course analyses were performed to examine glucose effects on the regulation of ASN1 mRNA. Results from Northern blot and RT-PCR analysis showed that low levels of glucose (0.1%, w/v) could abolish transcripts of ASN1 within 3 h (Fig. 2.4) and the degree of ASN1 repression was concentration dependent. These results suggested that ASN1 regulation by glucose was rapid at the transcriptional level. Although, glucose repression on ASN1 was rapid (3 h), derepression of ASN1 was slow (24 h). Light also could repress ASN1, but it took more than 24 h to obtain repression comparable to glucose (Fig. 2.5). Furthermore, light repression of ASN1 was blocked in a cell suspension culture incubated with photosynthesis inhibitor DCMU (3-(3,4-dichlorophenyl)-1,1-dimrthylurea) (Fujiki et al., 2001). Therefore, these results suggested that light repression of ASN1 was mediated through sugars produced by photosynthesis.

2.5.2 Cross-regulation of ASN1 by nitrogen and glucose

In plants, Asn is an important nitrogen storage and transport compound allocating nitrogen resources between source and sink organs. Although Gln-dependent Asn synthetase gene (ASN1) was repressed by exogenous sugars but induced additional Asn (Lam et al., 1994), it was not clear whether these regulations could be modulated by C/N
ratio. Results from different ratios of C/N suggested that \( \text{ASN1} \) is more sensitive to C than to N. I also tested nitrogen-free MS medium and lower concentration of glucose (0.01%, w/v) and higher concentration of Asn (10 mM). Preliminary results showed that neither inorganic nitrogen (60 mM), which included as macro-elements in MS salt nor supplementation of organic nitrogen Asn could restore the induction of \( \text{ASN1} \) gene in the presence of glucose.

2.5.3 Hexokinase is involved in the glucose repression of \( \text{ASN1} \)

To date several studies have proposed hexokinase as a sugar sensor by the phenotype of transgenic plants with altered levels of \( \text{AtHXK} \) and by using mannoheptulose, a specific inhibitor of HXK (Jang et al., 1997; Pego et al., 1999). Although it is still unclear how HXK functions as a sugar sensor, recent studies suggested that at least three glucose signal transduction pathways exist in plants on the basis of the results of gene expression analyses in HXK transgenic plants: \( \text{AtHXK1} \)-dependent pathway (Jang et al., 1997; Xiao et al., 2000), HXK-independent pathway (Martin et al., 1997; Mita et al., 1997b; Roitsch, 1999; Xiao et al., 2000; Ciereszko et al., 2001), and glycolysis-dependent pathway that depends on the catalytic activity of HXK (Xiao et al., 2000). The down-regulation of \( \text{ASN1} \) by glucose is probably mediated via HXK-dependent pathway, because only phosphorylatable forms of sugars such as glucose and mannose suppressed \( \text{ASN1} \) mRNA (Fig. 2.9). In addition, in the absence of glucose, \( \text{ASN1} \) was down-regulated in \( \text{HXX1} \) overexpressor 35S:AtHXK1 compared to the WT (Fig. 2.10). Transgenic \textit{Arabidopsis} plants over-expressing \textit{AtHXK} showed hypersensitivity to glucose, whereas the under-expressing \textit{AtHXK} showed hyposensitivity
to glucose (Jang et al., 1997). Thus, it implicated that even in the absence of exogenous glucose, \( ASN1 \) was repressed by endogenous glucose in \( 35S:AtHXK1 \) plants. Interestingly, \( gin2 \) (\( AtHXK1 \) knockout mutant) showed similar levels of \( ASN1 \) expression to that of in the WT (Landsberg \( erecta \), Ler), which could be ascribed to \( AtHXK2 \) might be compensated for the function in \( AtHXK1 \) (Fig. 2.9) (Jang et al., 1997).

### 2.5.4 \( ASN1 \) repression by sugar is unlikely affected by ABA

Recent genetic analyses of sugar-signaling mutants suggested that phytohormone ABA plays an important role in sugar-mediated gene expression (Arenas-Huertero et al., 2000; Finkelstein and Lynch, 2000a; Huijser et al., 2000; Laby et al., 2000; Rook et al., 2001). However, it is unclear the underlying mechanisms of integration between sugar signal and ABA signal. Also, unclear is the role of ABA in sugar signaling pathway. Fluridone has been used as a inhibitor of ABA biosynthesis (Moore and Smith, 1984; Lang and Palva, 1992). Our results showed the repression still occurred even in high concentration of fluridone (100 \( \mu \)M) (Fig. 2.11). This result suggested that \( ASN1 \) repression by glucose was not mediated through ABA under the conditions tested. Interestingly, ABA could enhance rather than repress \( ASN1 \) expression. Nevertheless, I showed that glucose repression of \( ASN1 \) persisted even in the presence of exogenous ABA (Fig. 2.11). Previous results showed anoxia, heat shock, and turgor stress (PEG) had no effect on maize \( AS \) mRNA (pZSS1), but copper stress, nitrogen deficiency and salt stress (NaCl) could upregulate \( AS \) transcripts in maize root tips (Chevalier et al., 1996). Therefore, induction of \( ASN1 \) mRNA by ABA might be connected to the stress effect but still need to be defined. To test this hypothesis further, I examined the
expression of *ASN1* expression by measuring the steady-state levels of *ASN1* transcript in known ABA biosynthetic or signaling mutants such as ABA biosynthetic mutant (*aba2*) and signaling mutant (*abi4*) (Fig. 2.13). As shown in Figure 2.13, the expression of *ASN1* mRNA was not modulated by exogenous glucose in these mutants. This data provided further evidence that *ASN1* repression by glucose was not affected by ABA under the conditions that I tested (Fig. 2.12). However, it is still not clear whether or not other ABA components could affect glucose repression of *ASN1* because I have yet to test all ABA mutants.

2.5.5 *ASN1* repression may not require de novo protein synthesis

Requirement of protein synthesis for the repression of *DIN6/ASN1* by sucrose was reported earlier using *Arabidopsis* suspension-cultured cells (Fujiki et al., 2000). To investigate the glucose effect on *ASN1* gene expression in *Arabidopsis* seedlings, I treated seedlings with 50 µM or 300 µM of cycloheximide and then seedlings were harvested up to 24 h after coincubation with glucose. Interestingly, my results indicated that CHX treatment with both concentrations delayed the downregulation of *ASN1* by glucose under 3 h (Fig. 2.14). This time-dependent regulation of *ASN1* in the presence of CHX implicated that *ASN1* repression by glucose might not require *de novo* protein synthesis and the delay was due to the effect of transcript stabilization. Conversely, it did only require cytoplasmic protein synthesis in a window of 3 h.
2.5.6 *Protein kinase is a positive regulator of de-repression of ASN1*

To determine whether protein phosphorylation or dephosphorylation events were involved in the expression of the *ASN1* gene, inhibitor of protein phosphatase type 2A (PP2A) okadaic acid or a general Ser/Thr protein kinase inhibitor K-252a was applied to the seedlings. Although okadaic acid did not affect the level of *ASN1* mRNA, K-252a strongly repressed *ASN1* expression in the sugar-starved seedlings (Fig. 2.15) suggesting that Ser/Thr protein kinases was a positive regulator of derepression of *ASN1*. Modulation by specific protein kinases/phosphatases was likely of critical importance in *ASN1* gene expression in plants under sugar starvation. In yeast, SNF1 Ser/Thr protein kinase was activated in response to glucose limitation and controls major signaling pathway for glucose repression (Carlson, 1999). Expression of an antisense, a plant homologue SnRK1 (SNF1-related protein kinase-1) sequence in the tubers of transgenic potato plants resulted in loss of sucrose inducibility of sucrose synthetase gene (Purcell et al., 1998). SnRK1 was also required for α-amylase gene expression in cultured wheat embryos and expressions of α-Amy1 and α-Amy2 were upregulated by sugar starvation (Laurie et al., 2003). However, no evidence showed that SnRK1 activity was regulated by glucose or other hexoses rather than sucrose (Halford and Hardie, 1998).

In summary, the *ASN1* regulation of gene expression has a unique mechanism that carries out specific and sensitive response to exogenous sugar. The *ASN1* is repressed by a low level of glucose (0.1%, w/v) in short time (3 h). In addition, *ASN1* repression involves sugar transport and phosphorylation, presumably by hexokinase. However, result shows *ASN1* repression may not require *de novo* protein synthesis for repression
with glucose. The results with pharmacological analysis using inhibitors show Ser/Thr protein kinase is a positive regulator of derepression of $ASN1$. Most critically, $ASN1$ repression by sugar is not affected by ABA. Further molecular, biochemical, and genetic analysis will provide valuable insight into the mechanisms used by plants to respond to the nutritional environment.
CHAPTER 3

CONSTRUCTION OF A FORWARD GENETIC SCREEN SYSTEM TO INVESTIGATE PLANT SUGAR SENSING AND SIGNALING

3.1 ABSTRACT

Mutants are pivotal tools for deciphering complex biological processes such as identifying the role of a gene or a protein complex in signal transduction. Forward and reverse genetic approaches have facilitated the discovery of gene function. At least twenty sugar-response mutants have been identified in *Arabidopsis* so far (Rolland et al., 2002; Leon and Sheen, 2003; Gibson, 2004). Although analyses of these sugar mutants have helped to discover several important components in sugar signaling pathway, it ended up with mutants carrying mutations in the same gene that were identified from several different screens. Most of these mutants were defective in either ABA- or ethylene-biosynthesis or response. To isolate specific sugar response mutants, I chose a screening strategy that selected *trans*-mutation that can affect the expression of a sugar sensitive marker gene,
*ASNI* (discussed in Chapter 2). For this purpose, *in vivo* reporter systems which consisted of the sugar-regulated *ASNI* promoter fused to either the luciferase coding sequence (*P*<sub>ASNI::LUC</sub> construct) or the green fluorescence protein coding sequence (*P*<sub>ASNI::GFP</sub> construct) were established. WT *Arabidopsis* plants have been transformed with reporter constructs carrying *ASNI* promoter fused with luciferase gene. Individual T<sub>3</sub> transgenic lines homozygous for each reporter construct were obtained. Among dozens of transgenic lines, two of them contained a single copy marker gene that displayed an expression pattern identical to the endogenous *ASNI* gene on the based on the expression levels of both RNA and protein. These lines were used for mutagenesis and mutant selection (to be discussed in Chapter 4).

### 3.2 INTRODUCTION

To date, a number of sugar response mutants have been isolated in *Arabidopsis*. These mutants were isolated by either sugar-mediated gene expression changes or sugar-dependent developmental phenotypes. For example, transgenic *Arabidopsis* plants harboring the light- and sugar-regulated plastocyanin promoter fused to the luciferase coding sequence (*PC-LUC* construct) were used for mutant screen (Dijkwel et al., 1997). In seedlings possessing a *PC-LUC* construct, both *PC* mRNA and luciferase activity levels were repressed by exogenous sucrose. Mutants defective in sucrose repression were identified by high luminescence levels in the presence of 3% (w/v) sucrose. The Sucrose-uncoupled (sun) mutants from these screens showed reduced luminescence in the
presence of sucrose (Dijkwel et al., 1997). Reduced sugar response (rsr) mutants were identified from progenies of the EMS mutagenized transgenic Arabidopsis plant containing the sugar and proline (Pro) inducible promoter, potato patatin class I (B33), fused to the GUS (Pat (B33)-iudA construct) (Martin et al., 1997). The analysis of the mutant rsr1-1 showed that sugar could not induce the patatin class I promoter and becomes hypersensitive to low levels of exogenous Pro, indicated possible link between carbon and nitrogen response (Hellmann et al., 2000). In addition, using the fusion constructs of the promoter of sugar responding Atβ-Amy gene encoding a β-amylase gene and GUS (Atβ-Amy:GUS) (Mita et al., 1995), low-level β amylase (lba) and high-level β amylase (hba) mutants were isolated. In the lba plants, the level of the mRNA for β-amylase after treatment with a 6% solution of sucrose, glucose or fructose was very low in leaves (Mita et al., 1997b). In contrast, the hba1 plant showed a high level of expression of Atβ-Amy in leaves of plants grown on medium with 2% glucose (Mita et al., 1997a). In addition, the 1997), hba1 plants showed increased levels of anthocyanin when plants were grown with 2% sucrose, suggested that sugar-induced accumulation of β-amylase and of anthocyanin share common regulatory mechanisms. Intriguingly, both lba1 and lba2 plants showed growth defect in low levels of sucrose (1%) could be caused by mutations on sugar-regulated genes that might be involved in growth and development (Mita et al., 1997a; Mita et al., 1997b). More recently, sugar-inducible promoter of ApL3 gene encoding a large subunit of ADP-Glc pyrophosphorylase (AGPase) was fused to a negative selection marker (ApL3::P450) (Rook et al., 2001). Transgenic Arabidopsis containing this ApL3::P450 construct survived the presence of the R7402 proherbicide only under non-inducing sugar conditions. M2 seeds from an EMS mutagenized
population were plated on media containing 100 mM sucrose and R7402 proherbicide. Seedlings that showed greening of the cotyledons were transferred and *impaired sucrose induction (isi)* mutants were screened (Rook et al., 2001). Complementation data suggested that *isi3* and *isi4* mutants are allelic to *abi4* and *aba2* respectively, but others (*isi1, isi2*) are not related to hormone-signaling pathways such as ABA or ethylene. Several *high sugar-response (hsr)* mutants have been isolated using the firefly luciferase cDNA under the control of the same *ApL3* promoter (Baier et al., 2004). While the *hsr* mutants exhibited relatively higher starch and anthocyanin levels, and lower chlorophyll levels in response to sugars than WT, physiological analysis data showed that these *hsr* mutations did not appear to have altered ABA and ethylene responses, altered sugar levels, or Glc uptake functions, suggested they may have altered sugar-specific responses (Baier et al., 2004). These mutant screens using relatively low sugar levels (30 mM sucrose) seem to reduce the probability of isolation of mutants resulting from osmotic effects of the sugar media (Rook and Bevan, 2003).

In the current research, I selected *ASN1* as a marker gene for screening to identify altered sugar response mutants (discussed in Chapter 2). Results of recent microarray analysis (Price et al. 2004) indicated that *ASN1* gene expression in glucose treated seedlings was highly repressed (-22.6 times) compared with non-treated seedlings. Because our strategy was to identify mutations causing abnormal responses to sugar, I have generated transgenic lines expressing *LUC* or *GFP* driven by *ASN1* promoter. These constructs could be used for mutant selection using low concentrations of sugar that do not induce osmotic responses that would normally cause by high concentrations of sugar.
3.3 MATERIALS AND METHODS

Plant materials and growth conditions

Wild-type and transgenic *Arabidopsis* lines were the Columbia-0 ecotype unless otherwise specified. Seedlings were grown in plates containing MS medium (Nitrogen, Carlsbad, CA) supplemented with 0.05% (w/v) MES (2-(N-morpholino) ethanesulfonic acid, Fisher Scientific, USA), resulted in pH 5.7 with 1M KOH, B5 vitamin, 0.7% (w/v) phyto-aga (Invitrogen, Carlsbad, CA), and sugar as indicated in each experiment For *in vitro* growth seeds were surface-sterilized with 95% (v/v) ethanol for 30 sec, and then with 50% (v/v) commercial bleach (Clorox) for 15-20 min, finally being washed with sterile distilled water. After a 3 d cold treatment at 4°C in the dark, seeds were spread on plates and left at 4°C for another 4 d synchronize the germination. The plates were incubated at 22°C under constant illumination (90 μmol of photon m² s⁻¹) for 6-7 days.

Construction of the parental line containing *ASN1* inducible promoter fused separately to both the *LUC* and *GFP* markers.

To make the fusion construct, the *ASN1* promoter was isolated by PCR using WT (Columbia, Col-0) *Arabidopsis* genomic DNA as a template. The *ASN1* promoter fragment (1.0 kb or 2.0 kb) with 47 bp coding sequence was fused with the reporter gene *LUC* and the cassette was inserted into the plant transformation vector pBI101.3 (Clontech, Palo Alto, CA). The second set of constructs was made by fusing the *ASN1* promoter (148 bp, 1.0 kb, or 2.0 kb) and full length *ASN1* coding sequence with GFP (see
Fig. 3.2). These constructs were used for the investigation of the effects of posttranslational modification on glucose signaling pathway. Arabidopsis WT (Col-0) plants were transformed with these constructs by Agrobacterium floral-dip method (Clough and Bent, 1998). Individual T3 transgenic lines homozygous for each reporter construct were obtained by drug selection (kanamycin, 50 mg L\(^{-1}\)) and production in the greenhouse.

\(P_{ASN1::LUC}\) transgenic plants:

The –1 kb to + 47 bp (1.075 kb) and –2kb to + 47 bp (2.060 kb) of genomic DNA fragments of \(ASN1\) were obtained by PCR using the following pairs of primers containing restriction enzyme sites (\(XbaI/NcoI\) to the 5’ end: 5’- GCTCTAGACCAAAATAGG\(CTT\)\(AAC\)\(TAT\) -3’ (-1kb forward), 5’- GCT\(\ CTCTAGACCAAAATAGG\(CTT\)\(AAC\)\(TAT\)\(AGG\)\(CTT\)\(AAC\)\(TAT\)\(AGG\) -3’ (-2kb forward), and 5’- CAT\(\ GCC\)\(ATG\)\(GCC\)\(TGA\)\(GAA\)\(TCA\)\(TCG\) -3’ (reverse).

\(P_{ASN1::GFP}\) transgenic plants:

The 1.9 kb, 2.75 kb, and 3.75 kb of the DNA fragments of \(ASN1\) containing putative promoter and coding sequence were obtained from Arabidopsis genomic DNA by PCR with the following pairs of primers containing restriction enzyme sites (\(SmaI/BspHI\)) at 5’ end: 5’- TCC\(\ CCC\)\(GGG\)\(TTT\)\(CTC\)\(TTC\)\(CTG\)\(GAC\)\(ATC\) -3’ (-148 bp forward), 5’- TCC\(\ CCC\)\(GGG\)\(ATC\)\(CAA\)\(AAT\)\(AGG\)\(CTT\)\(AAC\)\(TCT\)\(ACT\)\(AAT\)\(TCA\)\(ATC\)\(CTG\)\(GAC\)\(ATC\) -3’ (-1 kb forward), 5’- TCC\(\ CCC\)\(GGG\)\(ATC\)\(CAA\)\(AAT\)\(AGG\)\(CTT\)\(AAC\)\(TCT\)\(ACT\)\(AAT\)\(TCA\) -3’ (-2 kb forward), and 5’- CAT\(\ GTC\)\(ATG\)\(AAC\)\(TGA\)\(ATC\)\(ACA\)\(ACT\)\(CCT\)\(TCT\)\(ACT\)\(AAT\)\(TCA\)\(ATC\)\(TCA\)\(GAA\) -3’ (reverse). All amplification reactions
were conducted at 94°C for 30 sec, 50 °C for 45 sec, and 72 °C for 1min for 36 cycles, and 2 µl of the PCR products were loaded on a 1% agarose gel for verification. The DNA fragments were subcloned into a binary vector containing the firefly LUC coding sequence. DNA fragments for GFP constructs replaced with 35S promoter in sGFP vector (this vector was kindly provided by Dr. Yasuo Niwa, University of Shizuoka, Japan) then subcloned into the binary vector (pBI101.3). DNA sequencing and restriction enzyme digestion were carried out to confirm that the coding regions were in frame with the reporter gene. Primers used to verify the promoter/reporter region were were 5’-CCTGGACATCTGTCTGTT-3’ (ASN1/LUC) and 5’-AGAAGTCGTGCTGCTTCA-3’ (ASN1/GFP).

**Agrobacterium-mediated transformation of Arabidopsis.**

PASN1:: LUC and PASN1:: GFP were moved into Agrobacterium tumefaciens strain GV3101 (pMP90RK) by the electroporation-transformation and introduced into Arabidopsis thaliana plants (ecotype Columbia-0) by floral-dip method (Clough and Bent, 1998) or floral-spray transformation (Chung et al., 2000). A liquid culture (1 L/construct) carrying a construct was grown at 28 °C in LB medium with antibiotics gentamicin (15 mg L⁻¹), kanamycin (30 mg L⁻¹), and rifampicin (150 mg L⁻¹) to select for the binary plasmid and Agrobacterium strain. When OD₆₀₀ reached about 0.8, cells were spun down and resuspended in 5% (w/v) sucrose solution. Silwet L-77 (Lehle seeds, Round Rock, TX) was added to the cell suspension to a concentration of 0.05% (v/v). For floral-dip transformation, six week-old, bolting plants were used. For floral-spray transformation, 6-week old plants were sprayed three times at 8 h interval. After
transformation, the plants were covered with Saran wrap for 24 h to maintain humidity. The primary transformants (T₀) were allowed to grow and set seeds. T₁ seedlings were selected for growth on MS medium containing 50 mg L⁻¹ kanamycin for two weeks, and allowed to self-fertilize and set seeds after being transferred to soil. Two weeks later, T₂ plants were screened for kanamycin resistance. The segregating 75% kanamycin-resistant were allowed to self-fertilize and seeds were collected. T₃ lines showing 100% kanamycin-resistant seedlings were confirmed as homozygous. Lines with a single insertion that showed consistent normal glucose response from both endogenous *ASN1* and reporter gene were chosen for subsequent experiments. The bulk of these seeds were mutagenized with ethyl methanesulfonate (EMS) or activation-tagging mutagenesis (discussed in Chapter 4).

**Southern blot hybridization**

Genomic DNA was prepared (Sambrook et al., 1989) from 2-3 week-old soil-grown plants. Genomic DNA (10 μg) was digested with 100 units of *Bam* HI, *Eco* RI, *Hind* III, and *Xba* I with 5 μl 10x buffer (NEB #2), 0.5 μl 100x BSA for 14 h with reaction mix at 37 °C. Five micrograms each of digested DNA samples were separated on 0.8% agarose gel. After the sequential steps of acid depurination, denaturation, and neutralization, DNA was blotted onto nylon membrane and fixed by UV-crosslinking. Radiolabeled DNA probes were prepared by PCR reactions with 20 μl mixture containing 1x labeling buffer, 0.2 mM deoxynucleotide triphosphate (without ATP), 0.25 units of *Taq* polymerase (New England Biolab, Beverly, MA), 0.05 μg primers and 0.1 μg template DNA (pBI101-*ASN1::LUC*), and 1 μCi ³²P-α-ATP. Forty cycles of PCR
amplification were carried out at 94°C for 30 sec (denaturation), 50°C for 45 sec
(annealing) and 72°C for 60 sec (synthesis). Primers used for Southern blot analysis were
LUC-F, 5’- T GCCAATTTGC GCTGCT -3’ and LUC-R, 5’-
GTGAAAGTGCCAAACGC -3’. That membrane was hybridized for 12-16 hours in a
rotating hybridization oven (Robbins Scientific, Sunnyvale, CA) and washed in 500 ml of
2x SSC containing 1% (w/v) SDS for 15 min at room temperature, then washed 3 times
with 0.5x SSC containing 1% (w/v) SDS for 30 min at 60°C, then subjected to
autoradiography.

**Bioluminescence image analysis using CCD**

The substrate of luciferase enzyme, luciferin (D-Luciferin Firefly, potassium salt,
synth.) was purchased from Biosynth (Naperville, IL). A 100 mM stock solution was
prepared by dissolving it in distilled water and storing it at –80 ºC by aliquot of 500 µl in
1.5 ml microcentrifuge tubes. Then, the tubes were covered with aluminum foil to
prevent light exposure to prevent photo-oxidation. A 1 mM luciferin working solution
was freshly prepared in each experiment by adding distilled water and Triton X-100 (0.01
% (v/v), final concentration) and mixed well. The image system consisted of a CCD
camera (C-2400, Argus system, Hamamatsu, Bridgewater, NJ), a camera controller
(Argus-20 image processor), RGB monitor, and a computer with Adobe Photoshop®
software. Bioluminescence was collected for 45 sec using the image system consisting of
the CCD camera. The bioluminescence image were obtained after subtracting the random
noise from background. Photon counts were measured using the Hamamatsu Argus-20
image processor.
Isolating and mapping the parental lines

Seedlings were cultured as described above. The thermal asymmetric interlaced (TAIL)-PCR was performed to map the T-DNA insertion site in three transgenic lines (#174, #244, and #284) (Liu et al., 1995) using the degenerated primer AD1 (64-fold degeneracy), AD2 (128-fold degeneracy), AD3 (256-fold degeneracy), and nested primers as shown on Table 3.1. First TAIL-PCR reactions (20 µl) contained 2 µl 10x ExTaq buffer, 1µl 40 ng µg⁻¹ genomic DNA, 1.6µl 2.5mM dNTPs, 0.4 µl 10 pmol µl⁻¹ pBI101-L1, 1µl 100 pmol µl⁻¹ AD3 (degenerate primer), 0.2 µl 5 unit µl⁻¹ ExTaq polymerase (Takara, Madison, WI), and 13.8 µl distilled water. The second and third reaction mixtures are as follows:

2nd PCR (total 20 µl):
1.00 µl 1st PCR product (DF=50-200)
2.00 µl 10x ExTaq buffer
1.60 µl 2.5 mM dNTPs
0.40 µl 10 pmolµl⁻¹ pBI101-L2
0.80 µl 100 pmol µl⁻¹ AD3 (degenerate primer)
0.16 µl 5 U µl⁻¹ ExTaq polymerase
14.04 µl distilled water
3rd PCR (total 20 µl):
1.00 µl 2nd PCR product (DF=50-200)
2.00 µl 10x ExTaq buffer
1.60 µl 2.5 mM dNTP
0.60 µl 10 pmol µl⁻¹ pBI101-L3
0.60 µl 100 pmol µl⁻¹ AD3 (degenerate primer)
0.10 µl 5 U µl⁻¹ ExTaq polymease
14.10 µl distilled water

DNA sequencing of TAIL-PCR products

The TAIL-PCR reactions that generated fewer and thicker bands were selected and subcloned into pGEM-T Easy Vector® (Promega, Wisconsin, USA). Positive clones were confirmed with PCR using SP6 or T7 primer with T-DNA border primer, which were used in TAIL-PCR, R2 or L2. The plasmid DNA was purified by mini-prep kit (Qiagen, Valencia, CA) and sequenced. All sequences were blast searched against publicly available sequences from GenBank (http://www.ncbi.nlm.nih.gov/BLAST/) and further confirmed by TAIR (http://www.Arabidopsis.org/Blast/) database.

Analysis of sugar sensitivity in transgenic plants

Transgenic lines of the T3 generation homozygous for the transgene were used for the analysis of sugar sensitivity. About 10 seeds of the each transgenic and the control (wild-type) line were germinated and grown on different concentrations of glucose
(sugar-free, 1, 2, 3, 4, or 5% (w/v)). Plates were set vertically and the seeds allowed to

<table>
<thead>
<tr>
<th>Name</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>AD1</td>
<td>NTC GAS TWT SGW GTT</td>
</tr>
<tr>
<td>AD2</td>
<td>NGT CGA SWG ANA WGA A</td>
</tr>
<tr>
<td>AD3</td>
<td>WGT GNA GWA NCA NAG A</td>
</tr>
<tr>
<td>pBI-L1</td>
<td>CGTCTC ACT GGT GAA AAG AAA</td>
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<tr>
<td>pBI-L2</td>
<td>AGT ACA TTA AAA ACG TCC GCA A</td>
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<tr>
<td>pBI-R2</td>
<td>GCT CAT GAT CAG ATT GTC GTT T</td>
</tr>
<tr>
<td>pBI-R3</td>
<td>GTC ATA ACG TGA CTC CCT TAA</td>
</tr>
</tbody>
</table>

Table 3.1. Primers for TAIL PCR.

Abbreviations: N = A,C,T, or G; S = C or G; W = A or T.

The locations of each primer on T-DNA sequence are given in Figure 3.9.
<table>
<thead>
<tr>
<th>Reaction</th>
<th>Number of cycles</th>
<th>Thermal settings</th>
</tr>
</thead>
<tbody>
<tr>
<td>First PCR</td>
<td>1</td>
<td>94ºC, 1 min, 95ºC, 1 min</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>94ºC, 1 min, 65ºC, 1 min, 94ºC, 1 min, 72ºC, 3 min</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>94ºC, 1 min, 30ºC, 1 min, ramping to 72ºC over 3 min, 72ºC, 3 min</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>94ºC, 30 sec, 62ºC, 1 min, 72ºC, 3 min</td>
</tr>
<tr>
<td></td>
<td></td>
<td>94ºC, 30 sec, 68ºC, 1 min, 72ºC, 3 min</td>
</tr>
<tr>
<td></td>
<td></td>
<td>94ºC, 30 sec, 44ºC, 1 min, 72ºC, 3 min</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>72ºC, 5 min</td>
</tr>
<tr>
<td>Second PCR</td>
<td>1</td>
<td>94ºC, 1 min, 95ºC, 1 min</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>94ºC, 30 sec, 64ºC, 1 min, 72ºC, 3 min</td>
</tr>
<tr>
<td></td>
<td></td>
<td>94ºC, 30 sec, 44ºC, 1 min, 72ºC, 3 min</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>72ºC, 5 min</td>
</tr>
<tr>
<td>Third PCR</td>
<td>1</td>
<td>94ºC, 1 min, 95ºC, 1 min</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>94ºC, 30 sec, 64ºC, 1 min, 72ºC, 3 min</td>
</tr>
<tr>
<td></td>
<td></td>
<td>94ºC, 30 sec, 44ºC, 1 min, 72ºC, 3 min</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>72ºC, 5 min</td>
</tr>
</tbody>
</table>

Table 3.2. Cycle setting used for TAIL-PCR.

*Each segment of cycles consists of two high-stringency and one low-stringency.
germinate and grow in constant white light (90 µE m⁻² sec⁻¹) for 5 d. For phenotypic analysis, the images were recorded using digital camera (Canon, Lake Success, NY). For the comparison of the activities of luminescence, about 81 (9x9) seedlings were grown on the different concentrations of glucose (sugar-free, 1, 2, and 3% (w/v)) for 6 d under continuous light. They were then subjected to dark conditions for 28 h. The luciferase activity was measured by PC (Photon Counting) mode in Argus-20 image processor (Hamamatsu, Bridgewater, NJ) with CCD camera. All experiments were repeated at least twice.

3.4 RESULTS

3.4.1 Promoter analysis

To isolate components of the sugar-signaling pathway regulating sugar repression of _ASN1_ gene, fusion constructs containing promoter of _ASN1_ and _in vivo_ reporter were made. Although promoters of _AS_ in peas were identified by the deletion analysis and the gain-of-function experiments (Ngai et al., 1997), promoters in other species including _Arabidopsis_, were not clear. To identify _cis_-acting DNA elements in _ASN1_, up to -2 kb upstream region from start codon was analyzed by GenePalette software (University of California, San Diego, La Jolla, CA). Manual and software were downloaded from the GenePalette homepage (http://www.genepalette.org). Database was constructed by extracting 392 plant _cis_-elements from databases PLACE (plant _cis_-acting regulatory
Figure 3.1. *Cis*-element analysis of *ASN1* promoter. The “▲” and “▼” indicate sugar response-related *cis*-elements on forward (+) and reverse (-) directions, respectively. The #244 T-DNA insertion position is marked by “v”.

(Continued)
Figure 3.1 continued
<table>
<thead>
<tr>
<th>Cis element ID</th>
<th>Cis-element and position (bp)</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABRELATERD1</td>
<td>ACGTGG-1246, -554, -351, -164 (+); -1583, -1327, -555, -352 (-)</td>
<td>ABRE-like sequence (from -199 to -195) required for etiolation-induced expression of erd1 (early responsive to dehydration) in <em>Arabidopsis</em></td>
</tr>
<tr>
<td>ABREOSRAB21</td>
<td>ACGTSSSC-1582 (+)</td>
<td>&quot;ABA responsive element (ABRE)&quot; of wheat Em and rice (O.s.) rab21 genes; Proposed consensus sequence for the repeated motif (Em1a and Em1b) of wheat Em gene; S=C/G</td>
</tr>
<tr>
<td>ASF1MOTIFCAMV</td>
<td>TGACG-1882, -814 (+); -1749, -1325, -504, -437 (-)</td>
<td>&quot;ASF-1 binding site&quot;; TGACG motifs are found in many promoters and are involved in transcriptional activation of several genes by auxin and/or salicylic acid</td>
</tr>
<tr>
<td>ATHB6COREAT</td>
<td>CAATTATTA-1639 (+)</td>
<td>Consensus binding sequence for <em>Arabidopsis</em> homeodomain-leucine zipper protein, ATHB6; ATHB6 is a target of the protein phosphatase ABI1 and regulates hormone responses</td>
</tr>
<tr>
<td>CACGTGMOTIF</td>
<td>CACGTG-555, -352 (+ and -);</td>
<td>&quot;CACGTG motif&quot;; &quot;G-box&quot;; Found in light-responsive genes; Essential for expression of beta-phaseolin gene during embryogenesis in bean, tobacco, <em>Arabidopsis</em></td>
</tr>
<tr>
<td>CATATGGMSAUR</td>
<td>CATATG 905 (+ and -)</td>
<td>Sequence found in NDE element in soybean (G.m.) SAUR (Small Auxin-Up RNA) 15A gene promoter; Involved in auxin responsiveness</td>
</tr>
</tbody>
</table>

Table 3.3. Selected *cis*-elements and their known functions in *ASN1* promoter.

* Annotation is based on the information from PLACE database (http://rarge.gsc.riken.go.jp/cdna/promoter/).

Abbreviations: R = A or G; Y = C or T; W = A or T; S = C or G; and N = A, C, T, or G.

(Continued)
Table 3.3 continued

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<th>Cis element ID</th>
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<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPBFCOREDCDC3</td>
<td>ACACNNG -1453, -1150, -1073, -556, -353 (+); -555 (-)</td>
<td>A novel class of bZIP transcription factors, DPBF-1 and 2 (Dc3 promoter-binding factor-1 and 2) binding core sequence; Found in the carrot (D.c.) Dc3 gene promoter; Dc3 expression is normally embryo-specific, and also can be induced by ABA; The Arabidopsis abscisic acid response gene ABI5 encodes a bZIP transcription factor; abi5 mutant have a pleiotropic defects in ABA response; ABI5 regulates a subset of late embryogenesis-abundant genes; GIA1 (growth-insensitivity to ABA) is identical to ABI5</td>
</tr>
<tr>
<td>ELRECOREPCRP1</td>
<td>TTGACC -1990 (+)</td>
<td>EIRE (Elicitor Responsive Element) core of parsley (P.c.) PR1 genes; consensus sequence of elements W1 and W2 of parsley PR1-1 and PR1-2 promoters</td>
</tr>
<tr>
<td>GT1CONSENSUS</td>
<td>GRWAAW -1616, -1427, -1334, -948, -780, -672, -671, -600, -467, -63, -62 (+); -1733, -1563, -1290, -628, -157, -116, -1783, -1740, -1732, -653, -652, -52, -26 (-)</td>
<td>Consensus GT-1 binding site in many light-regulated genes, e.g., RBCS from many species, PHYA from oat and rice, spinach RCA and PETA, and bean CHS15; R=A/G; W=A/T</td>
</tr>
<tr>
<td>IBOX</td>
<td>GATAAG -499 (+); -634, -318 (-)</td>
<td>&quot;I-box&quot;; Conserved sequence upstream of light-regulated genes; Sequence found in the promoter region of rbcS of tomato and Arabidopsis; I box</td>
</tr>
</tbody>
</table>

(Continued)
<table>
<thead>
<tr>
<th>Cis element ID</th>
<th>Cis-element and position (bp)</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>IBOXCORE</td>
<td>GATAAA -1685, -1616, -948, -600, -560, -499, -467 (+); -1782, -1699, -1562, -1289, -633, -329, -317 (-)</td>
<td>&quot;I-box&quot;; Conserved sequence upstream of light-regulated genes; Conserved sequence upstream of light-regulated genes of both monocots and dicots</td>
</tr>
<tr>
<td>MYBCORE</td>
<td>CNGTTR -1757, -312 (+); -1603 (-)</td>
<td>Binding site for all animal MYB and at least two plant MYB proteins ATMYB1 and ATMYB2, both isolated from Arabidopsis; ATMYB2 is involved in regulation of genes that are responsive to water stress in Arabidopsis</td>
</tr>
<tr>
<td>MYBGAHV</td>
<td>TAACAAA -1084 (-)</td>
<td>Central element of gibberellin (GA) response complex (GARC) in high-pI α-amylase gene in barley (H.v.); GARC consist of the pyrimidine, TAACAAA and TATCCAC boxes</td>
</tr>
<tr>
<td>MYCATRD22</td>
<td>CACATG -1192, -1072 (+); -1218, -1190 (-)</td>
<td>Binding site for MYC (rd22BP1) in Arabidopsis (A.t.) dehydration-responsive gene, rd22; MYC binding site in rd22 gene of Arabidopsis thaliana; ABA-induction</td>
</tr>
<tr>
<td>NTBBF1ARROLB</td>
<td>ACTTTA -653 (+); -946, -72 (-)</td>
<td>NtBBF1(Dof protein from tobacco) binding site in Agrobacterium rhizogenes (A.r.) rolB gene; Found in regulatory domain B (-341 to -306); Required for tissue-specific expression and auxin induction</td>
</tr>
<tr>
<td>PYRIMIDINEBOXHV</td>
<td>TTTTTTCC -672 (-)</td>
<td>&quot;Pyrimidine box&quot; found in the barley (H.v.) EPB-1 (cysteine proteinase) gene promoter; Located between -120 to -113; Required for GA induction.</td>
</tr>
</tbody>
</table>
Table 3.3 continued

<table>
<thead>
<tr>
<th>Cis element ID</th>
<th>Cis-element and position (bp)</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>PYRIMIDINEBOXOSR AMY1A</td>
<td>CCTTTT -676 (-)</td>
<td>Pyrimidine box found in rice (O.s.) α-amylase (RAmy1A) gene; Gibberellin-response cis-element of GARE and pyrimidine box are partially involved in sugar repression (Morita et al., 1998).</td>
</tr>
<tr>
<td>SURE1STPAT21</td>
<td>AATAGAAAAA -1739 (-)</td>
<td>Sucrose Responsive Element (SURE); A motif conserved among genes regulated by sucrose (Grierson et al., 1994).</td>
</tr>
<tr>
<td>TATCCAOASAMY</td>
<td>TATCCA -1543, -1288, -1030, -489 (+); -1155 (-)</td>
<td>&quot;TATCCA&quot; element found in α-amylase promoters of rice (O.s.) at positions ca.90 to 150bp upstream of the transcription start sites; Binding sites of OsMYBS1, OsMYBS2 and OsMYBS3 which mediate sugar and hormone regulation of α-amylase gene expression (Toyofuku et al., 1998).</td>
</tr>
<tr>
<td>TATCCAYMOTIFOSR AMY3D</td>
<td>TATCCAY -489 (+); -1161 (-)</td>
<td>&quot;TATCCAY motif&quot; found in rice (O.s.) RAmy3D α-amylase gene promoter; Y=T/C; a GATA motif as its antisense sequence; TATCCAY motif and G motif are responsible for sugar repression (Toyofuku et al., 1998).</td>
</tr>
<tr>
<td>WBOXATNPR1</td>
<td>TTGAC -1990, -815 (+); -436 (-)</td>
<td>&quot;W-box&quot; found in promoter of Arabidopsis thaliana (A.t.) NPR1 gene; Located between +70 and +79 in tandem; They were recognized specifically by salicylic acid (SA)-induced WRKY DNA binding proteins.</td>
</tr>
</tbody>
</table>
DNA elements, http://www.dna.affrc.go.jp/htdocs/PLACE/) and AGRIS (Arabidopsis Gene Regulatory Information Server, http://Arabidopsis.med.ohio-state.edu/) database. Cis-elements involved in hormones, stresses, and light regulation were further selected and -2 kb upstream of *ASN1* gene was analyzed with GenePalette. The analysis results were confirmed by comparing them with the results by using databases on PLACE and AGRIS. Several cis-elements were found within -2 kb upstream region of *ASN1*, these included ABA, light, hormones, and sugar related functions (Fig. 3.1 and Table 3.3). Interestingly, while cis-elements involved in light and ABA regulation were dispersed along the selected promoter region, cis-elements involved in sugar regulation were mostly found at upstream region of -1 kb (Table 3.3).

### 3.4.2. Vector construction

To screen for mutants with abnormal sugar responses, transgenic *Arabidopsis* lines that expressed the firefly luciferase (LUC) or green fluorescence protein (GFP) under control of the *ASN1* promoter were generated (Fig. 3.2). For fusion construct containing LUC, two different upstream sequence regions (-1 kb and -2 kb) with LUC reporter were selected and subcloned into binary vectors (pBI101.3, Clonetech, Palo Alto, CA). For tagging with GFP, three upstream sequence regions (-148 bp, -1 kb, and -2 kb) plus coding sequence regions were selected and subcloned into the binary vectors (pBI101.3) (described detail in “Materials and Methods”). The length of the promoter used in the experiment was determined by cis-element analysis as well as by a previous study of pea *asparagine synthesis (ASI)* promoter in which it was shown that a minimal
Figure 3.2. Construction of $P_{ASN1}::LUC$ and $P_{ASN1}::GFP$ fusion genes. Two separate sets of constructs were made. A. Promoter fragment 1kb or 2kb upstream of $ASN1$ starting codon (ATG) plus 47 bp coding sequence was fused to LUC reporter. B. The promoter fragments 148 bp, 1 kb, or 2 kb upstream of ATG and $ASN1$ coding sequence without termination codon (TAA) were fused to GFP reporter. Restriction endonuclease cleavage sites shown are unique within T-DNA fragment, i.e. between right- and left-border sequences.
region of –148 bp is sufficient to confer sugar response (Ngai et al., 1997). For fusion constructs tagged bioluminescence luciferase gene, putative promoter region and coding sequence of \textit{ASN1} gene were fused just before the ATG initiation codon of LUC or GFP cDNA (Fig. 3.2). The finished plasmid DNA constructs were sequenced to confirm that the coding regions were in frame with the reporter.

### 3.4.3 Plant transformation

To estimate transformation efficiency, the transformants (\(T_0\)) were allowed to grow and set seeds and number of surviving \(T_1\) seedlings on MS salt medium containing 50 \(\mu\)g ml\(^{-1}\) were counted. Approximately, 0.3% (34 plants from app. 12,000 seeds) of plants survived on the selection medium. \(T_1\) seedlings were selected for growth on MS salt medium containing 50 \(\mu\)g ml\(^{-1}\) kanamycin for two weeks, and allowed to self-fertilize and set seeds after being transferred to soil. Figure 3.3 depicts the procedure of selecting lines containing transgene \(P_{\text{ASN1}}::LUC\).

### 3.4.4. Molecular and genetic analysis of transgenic plants

In order to determine the number of T-DNA insertions (encoding for kanamycin resistance and \(P_{\text{ASN1}}::LUC\)), segregation analysis was performed. The results showed that #17, #24, and #28 had 3 to 1 ratio of kanamycin-resistant (Kan') to kanamycin-sensitive (Kan\(^s\)) phenotype Chi-square test (Glantz, 2001) was performed to calculate deviation from the Mendelian ratio. The Chi-square values in three transgenic lines (#17, #24, and #28) were lower than the test values, which means that there was no statistically significant deviations from a 3:1 segregation ratio on the 5% level (Table 3.4). However,
the segregation ratio on #15 and #22 showed that these lines might have two inserts since
the ratios were greater than 3:1 (Rios et al., 2002). Among the dozens of progenies from
T$_2$ lines, homozygous T$_3$ lines that displayed 100% kanamycin-resistant were selected. I
found five homozygous transgenic lines (#154, #174, #224, #244, and #284) whose
reporter gene expression level was similar to the endogenous $ASN1$ gene (Table 3.4). It
was further confirmed that #244 and #284 to have single copy of T-DNA insertion and
their glucose repression of $ASN1$ was normal on both RNA and protein level (Table 3.4,
3.5, and Fig. 3.4). Interestingly, $LUC$ transcripts on #174 showed a very low level on the
blot. Therefore, #244 and #284 were used for mutagenesis and mutant selection
(discussed in Chapter 4).

As segregation for kanamycin resistance cannot be used as a sole criterion to
determine T-DNA copy, I performed Southern hybridizations on individual mutant plants
using $LUC$ fragment as a probe (Fig. 3.5). On the basis of restriction map (Fig. 3.2), the
selected restriction enzymes (BamHI, EcoRI, HindIII, and XbaI) would not cut $LUC$ gene. Thus,
plant containing single T-DNA copy may produce one major band. Result showed that all three
transgenic lines appeared to contain single copy of transgene because single dominant band
was observed in #174 (XbaI), #244 (XbaI), and #284 (EcoRI, HindIII, and XbaI) (Fig. 3.5).
Minor bands were likely to be non-specific as evidenced by the non-specific signals in maker
lanes. The non-specific minor bands may have been caused by incomplete DNA digestion
by restriction endonucleases. Alternatively, none of these three transgenic lines contain
single T-DNA copy. Each of the minor bands may represent an additional copy of T-DNA.
This possibility seems unlikely because it would be hard to explain why each line contains
multiple copy of T-DNA and yet all of them appear to have single copy on the basis of
Figure 3.3. Outline of the method to select transgenic lines homozygous of $P_{\text{ASN1}}::\text{LUC}$.

$T_0$, parental plants used for Agrobacterium-mediated transformation.
<table>
<thead>
<tr>
<th>Line Number§</th>
<th>Type</th>
<th>Total (N)</th>
<th>Kan $^r$</th>
<th>Kan $^s$</th>
<th>$\chi^2$†</th>
</tr>
</thead>
<tbody>
<tr>
<td>#15</td>
<td>T$_2$</td>
<td>279</td>
<td>255</td>
<td>24</td>
<td>30.83</td>
</tr>
<tr>
<td>#17</td>
<td>T$_2$</td>
<td>347</td>
<td>249</td>
<td>98</td>
<td>1.03</td>
</tr>
<tr>
<td>#22</td>
<td>T$_2$</td>
<td>359</td>
<td>326</td>
<td>33</td>
<td>40.07</td>
</tr>
<tr>
<td>#24</td>
<td>T$_2$</td>
<td>309</td>
<td>236</td>
<td>73</td>
<td>0.51</td>
</tr>
<tr>
<td>#28</td>
<td>T$_2$</td>
<td>359</td>
<td>267</td>
<td>92</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Table 3.4. Segregation analysis using $\chi^2$-test to estimate T-DNA copy number.

§#15 and #17 are progenies of transformants (T$_1$) carrying construct with 1kb promoter.

The #22, #24, and #28 are progenies of transformants (T$_1$) carrying construct with 2kb promoter.

†The $\chi^2$ is given for the ratio of 3:1 (Kan$^r$/Kan$^s$).

‡No significant deviation from the expected ratio at a level of $P = 0.05$. 
Table 3.5. Glucose repression of *ASN1* and reporter gene determined by RT-PCR and image analysis.

§Line numbers beginning with digit “1” and “2” are the plants carrying reporter gene with 1 and 2 kb promoter, respectively.

†Activities are represented by the following symbols:

(lower) – – < – < +/- < + < ++ (higher).

<table>
<thead>
<tr>
<th>Line number§</th>
<th><em>ASN1 mRNA</em></th>
<th><em>LUC mRNA</em></th>
<th>Luciferase image</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sugar-free</td>
<td>Glucose 0.5%</td>
<td>Sugar-free</td>
</tr>
<tr>
<td><strong>WT</strong></td>
<td>+†</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>#125</strong></td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>#154</strong></td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td><strong>#164</strong></td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>#174</strong></td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>#224</strong></td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td><strong>#244</strong></td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td><strong>#284</strong></td>
<td>+</td>
<td>–</td>
<td>+/-</td>
</tr>
</tbody>
</table>
RNA gel blot analysis was used to determine the levels of steady state mRNA of *ASN1* and *LUC*. The WT (Col-0) plants and transgenic plants were grown in MS liquid medium with 3% glucose in continuous light for 6 days. Plants were then washed 3 times with sugar and nitrogen free MS medium and dark adapted for 24 h to deplete internal sugar and nitrogen. Plants were then treated with or without 3% glucose in the dark for 3h in the dark before tissue collection and RNA extraction. Five micrograms of total RNA was loaded in each lane. Equal loading is indicated by uniform intensity of rRNA bands stained by ethidium bromide.
Figure 3.5. Southern blot analysis to determine T-DNA copy number.

Genomic Southern hybridization was performed using full length LUC cDNA as a probe. Genomic DNA from Arabidopsis WT (Col-0) and transgenic plants #174, #244, and #284 were digested with BamHI, EcoRI, HindIII, and XbaI. Five micrograms of genomic DNA were blotted onto nylon membranes and analyzed with Southern blot analysis. Numbers at left are molecular markers in kilo-bases (kb). Arrows (→) indicate major single bands.
Figure 3.6. Phenotypic analysis of the transgenic plants.

Ten seedlings each were grown on MS plates containing different concentrations of glucose. Plates were set vertically for the comparison of seedling growth under light for 5 days.
segregation ratio of kan\textsuperscript{R} gene, unless coincidentally there is only one copy of T-DNA contains intact kan\textsuperscript{R} gene in all three lines and the rest of the kan\textsuperscript{R} gene all get inactivated by deletion or silencing.

To investigate whether homozygous transgenic lines have any defects in plant development, seeds were sown on both sugar-free and 3\% (w/v) glucose containing MS plates. The results showed that all three transgenic lines had similar phenotype as wild-type throughout the life cycle (Figure 3.6). Therefore, #244 and #284 were used for mutagenesis and mutant selection.

3.4.4.1. Tissue specific expression of \textit{P}_{\text{ASN1}}::LUC

The \textit{P}_{\text{ASN1}}::LUC plants were used to determine tissue specificity of \textit{ASN1} expression (Figure 3.7B). In \textit{P}_{\text{ASN1}}::LUC transgenic plants (#244), the luciferase activity was high in shoot meristem and in the floral organs. The expression of luminescence was higher in young leaves than old leaves. Together these results suggest that \textit{ASN1} is preferentially expressed in sink tissues where sugar is mainly imported from the source tissues. The luciferase activities of these lines are given in Figure 3.7. Consistent with mRNA accumulation, luminescence intensity on #244 and #284 lines were negatively regulated by glucose. However, luminescence activity in #174 did not respond to glucose normally, which was similar to the Northern data (Fig. 3.4). By contrast, luminescence was still present in #284 in the presence of glucose although Northern blot analysis indicated a normal repression of \textit{LUC} mRNA accumulation (Table 3.5. and Fig. 3.4).
3.4.4.2 Molecular cloning of T-DNA inserted loci

The abnormal expression of $P_{\text{ASN1}}::LUC$ in two transgenic lines could be a result of insertional effect. To find out the locations of T-DNA insertion in the genome, a modified thermal asymmetric interlaced (TAIL)-PCR protocol was used to amplify sequences flanking the T-DNA insertions (Figure 3.3). Three successive amplification reactions were performed using nested primers complementary to known sequences (pBI101.1) and arbitrary (AD) primers. Typically, reactions with a T-DNA border primer (pBI101-R2, -R3, -L1, and -L2) and one of the three AD primers (AD1, -2, and -3) were used for each round of PCR to maximize the likelihood of generating a product (Tables 3.1, and 3.2, and Figure 3.9). The examples of TAIL-PCR (2nd PCR reaction) are given in Fig 3.10. Bands showing fewer and high intensity in a lane were isolated and subcloned into pGEM-T Easy Vector (Promega, Madison, WI). Ten finished plasmids were sequenced and the inserts from five samples (Fig. 3.10, marked as “$\circ$”) contained both T-DNA and Arabidopsis genomic sequence and others had either only T-DNA regions or no T-DNA inserts. These above five sequences originated four from first TAIL-PCR reaction (AD1) and one from third TAIL-PCR reaction (AD3) products (Fig. 3.10).

In #174, T-DNA was inserted into a region upstream of the tRNA-Met gene. The function of the tRNA-Met is not clear (Fig. 3.11). T-DNA in #244 was inserted into the exon region of $\beta$-carotene hydroxylase (GeneBank accession no. AB025606) (Fig. 3.12). Although the function of other $\beta$-carotene hydroxylase reported enhanced stress tolerance in Arabidopsis (Davison et al., 2002), the function of this particular $\beta$-carotene hydroxylase gene was unknown. Interestingly, based on the sequence analysis on the
Figure 3.7. *In vivo* expression of $P_{ASN1}::LUC$ in transgenic plants.

**A.** Image analysis of #244 grown on MS plates in the presence (3%) or absence of glucose for 7 days. Expression level is indicated by artificial color superimposed on the seedlings. Red represented the highest expression. **B.** Image analysis of #244 grown on soil in the greenhouse for 20 days. Because $P_{ASN1}::LUC$ is light repressible, plants were dark adapted for 2 days prior to the analysis.
Figure 3.8. Quantitative analysis of $P_{ASN1::LUC}$ expression.

Data were obtained from 7-day-old seedlings grown on MS plates with different concentrations of glucose. Quantitative luminescence intensity was obtained using photon counting tool in the Argus system package. Error bars represent SD from two replicates.
Figure 3.9. Specific primers used for TAIL-PCR and DNA sequencing reaction.

A. Schematic diagram of the T-DNA vector indicating the position of the specific primers with respect to the right border (RB) and the left border (LB).

B. Nucleotide sequences of specific primers R2, R3, L1, L2, and encompassing regions.
Figure 3.10. Examples of results from TAIL-PCR.

TAIL-PCR reactions generated bands. The fewer and thicker bands were selected to subclone into TA-vector. The sequencing data showed that the marked (△) bands contained franking region of T-DNA. Lanes 1, 5, and 9, WT; lanes 2, 6, and 10, #174; lanes 3, 7, and 11, #244; lanes 4, 8, 12, #284.
Figure 3.11. T-DNA insertion site in #174 transgenic line.

‘(LB-TDNA-RB)’ indicates a T-DNA is inserted in the promoter of *AtASN1* in chromosome V.
Figure 3.12. T-DNA insertion site in #244 transgenic line.

‘(LB-TDNA-RB)’ indicates T-DNA insertion site in chromosome V.
Figure 3.13. T-DNA insertion site in #284 transgenic line.

‘(LB-TDNA-RB)’ indicates T-DNA insertion site in chromosome III.
genomic DNA flanking the T-DNA right border (RB) in #284, T-DNA was found at -348 bp upstream region of ASNI in Arabidopsis chromosome III (Fig. 3.1 and Fig. 3.13). However, results here are not conclusive because I have only obtained the right border junction sequence and it not clear whether this junction sequence is derived from the authentic ASNI locus or perhaps it is a fragment of deleted, rearranged, and concatmerized T-DNA construct inserted into an unknown locus.

3.5 DISCUSSION

To dissect sugar signaling pathways, I have been employing a genetic screen system using promoters of ASNI with in vivo reporter GFP or LUC constructs. In the current research, LUC constructs were chosen for further experiment. Expression of the ASNI gene was highly responsive to external glucose, and therefore most suitable for identifying mutants affecting its glucose repressible expression. I transformed plants with T-DNA containing LUC reporter genes transcriptionally fused to the ASNI promoter. The use of the LUC reporter can help with the identification of trans-signaling mutations because cis-acting mutations can be identified via the difference in the expression pattern between endogenous (ASNI) and the reporter gene. In addition, the LUC assay has low background bioluminescence that allows high magnitude of sensitivity to detect the change of gene expression compared with the GUS assay in which slow protein turnover and lower sensitivity might be problematic for mutant screen
(Yamamoto and Deng, 1998). Although limited number of cis-elements conserved in sugar-responsive genes were known, the putative promoter region of \textit{ASN1} contained several cis-elements that are conserved in the regulation of hormones, light, stress, and sugar. Noticeably, a higher number of sugar-responsive motifs were found in the –2 kb promoter sequence of \textit{ASN1} (Fig. 3.1). Interestingly, I found that reporter gene responded more precisely to external sugar in plants containing reporter with 2kb promoter than those with 1 kb promoter (Table 3.3), indicating that some element(s) between –2kb and –1kb of the \textit{ASN1} promoter could be responsible for sugar response in the transgenic plants.

The results of genetic analysis indicated that three lines contained a single T-DNA insert (ratio Kan\textsuperscript{R}: Kan\textsuperscript{S} = 3:1) and two lines contained two or more inserts (ratio greater than 3:1). In some of the lines with two or more inserts, it has been known it is difficult to determine how many functional inserts are present (Koncz et al., 1992). As fewer inserts generally simplify genetic analysis, I selected the plants with single T-DNA insertion and functional reporter for further studies. These transgenic lines (T\textsubscript{2}) showed sugar response similar to the WT, suggesting that integration of T-DNA in their genome did not cause any altered sugar-dependent developmental processes (Fig. 3.6).

I examined the expression patterns of the \textit{P_{ASN1}}::\textit{LUC} expression in time and space in whole plant. \textit{P_{ASN1}}::\textit{LUC} was actively expressed in two major locations, the vegetative shoot meristem and the floral organs. Interestingly, in flowering state, 48 hr dark treatment on the transgenic plants lead to high expression in young leaves, not in old leaves. The MPSS (Massively Parallel Signature Sequencing) signature analysis (http://mpss.udel.edu/at) indicated that the \textit{ASN1} transcripts are highly localized in
inflorescence (243 TPM, transcripts per million), leaf (86 TPM), and silique (54 TPM).

These results are generally consistent with reporter gene expression in the transgenic line (#244) (Fig. 3.7)

As the reporter gene expression seemed to be abnormal in some transgenic lines, thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR) (Liu et al., 1995) was used to amplify unknown sequences adjacent to T-DNA insertion sites in *Arabidopsis* transgenic lines. Nested, insertion-specific primers were used together with arbitrary degenerate primers (AD primers), which were designed to differ in their annealing temperatures. Sequencing results have revealed T-DNA insertion sites in these three transgenic lines. In #174, T-DNA was inserted into the promoter region of tRNA-Met gene. Since in #174 the endogenous *ASN1* is regulated by glucose normally, it is assumed that the lack of de-repression of the *P_{ASN1}::LUC* in the absence of glucose is caused by unknown positional effect. In case of #244, insertion site of T-DNA was in exon sequence of b-carotene hydroxylase. My data suggest that this b-carotene hydroxylase is probably not involved in glucose signal transduction that regulates the expression of *ASN1* gene because neither sugar-dependent development nor sugar-dependent *ASN1* expression is altered in #244. Interestingly, #284 was inserted into the promoter of endogenous *ASN1* gene. Intriguingly, while the endogenous *ASN1* seems to respond to glucose normally (Fig 3.4), the reporter gene carrying the same promoter is defective in sugar response. On one hand the mRNA expression data suggest that #284 is defective in de-repression (no expression in the absence of glucose), on the other hand the protein expression based on the luminescence indicates that #284 is defective in glucose repression (Fig. 3.4 and Table 3.5). These results suggested that the integration
of additional copy of the promoter of \textit{ASN1} could have altered the responses in transcriptional and posttranscriptional levels. Alternatively, T-DNA in #284 could have inserted into an unknown locus. The TAIL-PCR product might have been a fragment of the T-DNA itself. The “false” junction could have been a result of deletion, rearrangement, and concatemerization of the T-DNA; this phenomenon is well documented in the literature (Kohli et al., 2003). Additional analyses such as DNA sequencing, DNA restriction mapping, and DNA gel-blot analyses are needed to unbiased determine the nature of the T-DNA insertion in #284. Because the genetic approach using transgenic \textit{Arabidopsis} containing \textit{P}_{\text{ASN1}}::\textit{LUC} is quantitative by virtue of luciferase and responds sensitively to sugar by virtue of \textit{ASN1}, I could isolate mutants more precisely responding to sugars by using #244.
CHAPTER 4

IDENTIFICATION OF MUTANTS WITH ALTERED GLUCOSE RESPONSE

4.1 ABSTRACT

To isolate mutants my genetic screen used the firefly luciferase reporter gene driven by the sugar-responsive $ASN1$ promoter. Results of initial analysis on transgenic lines suggested that the expression of $P_{ASN1}::LUC$ was highly localized in shoot tissues, thus these plant materials were suitable for identifying sugar signaling mutants because they would be accessible by imaging screen system. In the current research, transgenic lines ($T_3$) were mutagenized with EMS or activation-tagging vector. After a series of preliminary image analyses using various growth conditions and glucose treatments, I found optimal conditions for mutant screen.

A large number of seeds from each transgenic line were mutagenized. Several putative mutants have been isolated from mutagenized $M_2$ population. In addition to
abnormal glucose repression of \textit{ASN1} gene, some of them have major differences in the
growth and development, although further purification of these lines by backcross will be
highly required. Results here demonstrate that this mutant screen system is robust and it
has a high potential to uncover novel sugar signaling components.

\section*{4.2 INTRODUCTION}

Mutagenesis could be accomplished by chemical, physical, or molecular methods. Since each of these methods has unique advantages and drawbacks, I used
complementary methods, i.e., ethylmethane-sulphonate (EMS) and activation tagging.
One of the most effective ways to uncover gene functions is to inactivate the gene of
interest. T-DNA insertion mutagenesis is caused by the disruption of a gene by a
segment of the tumor-inducing (Ti) plasmid of \textit{Agrobacterium tumefaciens} flanked by
short imperfect repeat border sequences (known as right and left borders) that define the
boundaries of the T-DNA and virulence (\textit{vir}) genes. \textit{Agrobacterium tumefaciens}
transfers Ti plasmid to the nucleus of the host plant cell. The T-DNA insertion often
results in a complete loss of function (Krysan et al., 1999). T-DNA carrying a reporter
gene can facilitate the identification flanking sequence. Although T-DNA has many
advantages over traditional mutagenesis, low copy number and random insertion are also
known disadvantages of T-DNA insertion mutagenesis. T-DNA insertion mutagenesis
requires many more lines to obtain the same number of mutants and to cover the entire
genome. T-DNA insertion mutagenesis can create either gain-of-function or loss-of-
function mutants and the resulting mutants have a chance to be tagged. While insertional mutants offer obvious advantages in gene cloning, there are a number of limitations associated with transformation-mediated mutagenesis. For example, certain regions of the genome may be less accessible for the insertional inactivation, thus preventing a random coverage of the genome. To overcome this problem, mutageneses causing deletion or base pair change have been used in a number of organisms including Arabidopsis. Two popular methods, radiation mutagenesis and chemical mutagenesis, are being widely used.

Chemical mutagenesis often causes point mutation that could result in nonsense, missense, or silent mutation. Multiple alleles of EMS mutants can greatly facilitate the analysis of gene function due to the nature of the mutation. In chemical mutagenesis, a number of chemicals have been used to generate large mutant collections. The most used chemical mutagens are the alkylating agents. These chemicals react readily with purines, pyrimidines, and phosphates. The most preferred alkylating agent is ethylmethane-sulphonate (EMS). The main product of alkylation of nucleic acids is 7-alkyl guanine that can base pair with thymine yielding a G to A alteration but also T to C alterations by the formation of 4-ethyl-thymine. Another effect of alkylating agents is depurination that causes a gap in one of the nucleic acid strands and as a result, a deletion or a random incorporation of any of the four bases can occur during replication. Depurination can also lead to chromosome breaks if no nucleotide is introduced in the gap. EMS mutagenesis has been widely used for forward genetic screen.

Activation-tagging mutagenesis can discover genes that result in a lethal phenotype when mutated by loss-of-function mutagenesis. Furthermore, genes that have
a redundant function are unlikely to be discovered by loss-of-function mutant screen. This is due to the tremendously low probability of mutating multiple genes at the same time during mutagenesis (Nakazawa et al., 2003). However, if only one of the two redundant genes is activated during activation-tagging mutagenesis, a phenotype of the exaggerated function of the gene may be revealed. In fact, activation-tagging mutagenesis often generates loss-of-function mutants. Activation tagging uses a gene tag engineered to contain transcriptional enhancer sequences. Following the insertion of such a tag into the plant genome, expression of flanking sequences becomes deregulated, producing a dominant mutation. Therefore, mutant screens can be performed in the first generation (M1). When using activation-tagging, the second generation (M2) can be used to isolate loss-of-function mutants because insertions that reside within the coding region of the gene sometimes cause loss-of-function effects. The tetramerized cauliflower mosaic virus (CaMV) 35S promoter, can cause transcriptional activation of downstream gene.

In the current research, I used EMS to generate loss-of-function mutants and activation tagging was used to produce gain-of-function mutants from the homozygous glucose-repressible \( P_{ASN1}::LUC \) lines. The putative mutants exhibiting relatively high luciferase activity were identified in seedling population grown on media containing 3% (w/v) glucose. The putative mutants showed pleiotropic phenotypes including dwarf, early senescence, and leafy phenotype. Detailed molecular characterization of such mutants may lead to better understanding of the role of sugar-regulated gene expression network in cell division, cell elongation, development, and stress responses.
4.3 MATERIALS AND METHODS

Plant Materials and Growth Conditions

For mutant screen, seeds were surface-sterilized in 95% (v/v) ethanol for 1 min followed by 50% (v/v) commercial bleach (Clorox) for 15-20 min, and rinsed five times with sterile water. After a 3 d cold treatment at 4°C in the dark, approximately 500 seeds were plated on 1x basal Murashige and Skoog media (MS) containing 3% (w/v) glucose and left at 4°C for another 4 d to synchronize germination. For in vitro analysis, plants were grown under constant light (90 µmol m⁻² s⁻¹) at 22°C. Seedlings were then transferred to dark conditions for 1 d in a sugar-free medium to deplete endogenous sugars. Soil-grown plants were produced in the growth chamber under long-day (16h light/ 8h dark) conditions at 21°C until seeds were ready for harvesting.

EMS mutagenesis

Homzygous seeds of \( P_{ASN1::LUC} \) lines containing a single T-DNA locus were mutagenized by EMS (ethylmethan sulfonate). From the \( ASNI::LUC \) transformants (T\( _3 \)), #244 and #284 were selected for mutagenesis and seeds were bulked up. Approximately 80,000 seeds per lines were mutagenized with 0.3% EMS (methanesulfonic acid ethyl ester, Sigma-Aldrich, St. Louis, MO) for 12 h. Seeds were washed with distilled water 15 times over the course of 3 h. After washing, seeds were immediately sown at about one
seed per square cm. The mutagenized seeds (M\textsubscript{1}) were divided into 20 pools and grown in greenhouse to allow self-pollination to produce M\textsubscript{2} population.

To identify mutants with abnormal sugar-regulated expression of \textit{P\textsubscript{ASN1}::LUC}, 6-7 day-old seedlings were grown on MS plates with 3\% (w/v) glucose. The outline of the mutant screen procedure is given in Figure 4.1. Putative mutants were isolated from the secondary screen, and then transferred into soil and grown under 16 h light/8 h dark conditions in the growth chamber.

\textbf{Activation-tagging mutagenesis}

The activation-tagging vector, pSKI015 (GenBank accession no. AF187951, was kindly provided by Dr. Guo-Liang Wang, The Ohio State University) harboring BAR gene (confers Basta resistance) for selection in soil, constructed by Weigel lab (Weigel et al., 2000). I transformed pSKI015 into #244 and WT background as a control using the \textit{Agrobactrium} floral-dip method (Clough and Bent, 1998). Commercial Basta (Finale, glufosinate-ammonium, 5.78\%) was obtained from Farnam (Phoenix, AZ) and sprayed twice a week with 1x-diluted solution to select transgenic seedlings in soil.

\textbf{The impact of EMS mutagenesis on plant growth and development}

The EMS-mutagenized M\textsubscript{2} and the control (wild-type) plants were germinated and grown in constant white light (90 \textmu E m\textsuperscript{-2} sec\textsuperscript{-1}) in full-strength MS plates containing different concentrations of glucose (sugar-free, 1, 2, or 3\% (w/v)) for 5 d. Images were recorded using a digital camera (Canon, Lake Success, NY) and further processed using Adobe Photoshop\textsuperscript{®} software. Phenotypic analysis of putative mutants was done with 2-3
week-old plants grown in the growth chamber under long-day (16h light/ 8h dark) conditions at 21ºC.

**Bioluminescence image analysis**

For luciferase imaging, approximately 500 transgenic *Arabidopsis* seeds (M₂) were planted individually onto a 15mm Petri dish containing MS (Murashige and Skoog, 1962) 0.7 % (w/v) agar, 3% (w/v) glucose, and kanamycin (50 µg mL⁻¹) for 7 days. On the 7th day, the seedlings were then sprayed with a mixed solution of 1mM luciferin and 0.01% Triton X-100, 15 min before each imaging. Bioluminescence was collected for 45 sec using the image system with a CCD camera. Minimum values of the bioluminescence image were obtained from the background which was subtracted, creating minimum-subtract images devoid of random noise. These images were at the threshold with filters to remove plants that emitted wild-type luminescence levels in order to classify putative mutants. Putative mutants were identified by superimposing the LUC images onto the corresponding reference images and transferred to new glucose containing MS plates for the second screens and rescreened with the same procedure as described above. Finally, the putative secondary screened mutants were transferred into soil. M₃ were collected for rescreen and further analysis.
Figure 4.1. Outline of mutant screen.

The screen was carried out with mutagenized plants expressing the firefly luciferase reporter under control of sugar responsive promoter. After spraying the luciferase substrate, luciferin, a CCD camera was used to detect luminescence emitted by the plants. The putative sugar response mutants were transferred into soil for seed production.
4.4 RESULTS

4.4.1 Systematic mutagenesis

Approximately 80,000 seeds each of #244 and #248 were mutagenized with EMS. Although its glucose repression seems abnormal, #284 was included as backup due to its high level of luminescence. Indeed, #284 still has a glucose response, which could be distinguished by adjusting the threshold of the imaging system. Approximately 600,000 and 1,300,000 M$_2$ seeds were obtained from #244 and #284 respectively, after allowing the M$_1$ to self-pollinate in the greenhouse. Currently, 80,000 M$_2$ seedlings of #244 have been screened. The phenotypes of putative mutants from the screens included the high expression of $P_{ASN1}$::$LUC$ in the presence of sugar, delayed flowering, delayed senescence, and abnormal plant size. These lines will be required to back-cross at least three times with the parental $P_{ASN1}$::$LUC$ line to confirm these morphological phenotypes. The progenies will be reselected from the F$_2$ generation based on increased luciferase activity in 7 d old seedlings grown on media containing sugar.

I have initiated T-DNA activation tagging to isolate mutants involved in sugar signaling. About 300,000 seeds were generated from #244 for trial and screened in soil by spraying diluted (1x) herbicide Basta solution. About 0.3% T$_1$ seedlings (6 out of 2,000 seedlings) survived and were selected for further analysis. This translates to 900 independent lines and thus is unlikely to generate a high enough number of transgenic plants for standard mutant screens.
4.4.2 Mutant screens

Results of phenotypic analysis indicated that the EMS-mutagenesis was effective because approximately 20% of M<sub>2</sub> seeds were not able to germinate normally and many individuals displayed albino phenotypes (Fig 4.2). Severe developmental phenotype implicated that a high percentage of the M<sub>2</sub> might contain multiple mutations caused by EMS. A mutagenesis condition with maximum efficacy and minimum side effects could be determined by trial and error. A protocol using lower concentrations of EMS or shorter treatment time might avoid such problems. To date, approximately 80,000 M<sub>2</sub> seeds were screened and I found 50 putative mutants that exhibited abnormal expression of \( P_{ASN1}::LUC \).

On the primary screen, about 500 seeds (M<sub>2</sub>) were spread on 3% (w/v) glucose containing MS plates and screened using a luminescence imaging system. Putative mutants were selected based on luminance levels, which had more than 600 relative luminescence unit (RLU) corresponded to the stringency that approximately excluding plants included in 5% background level, and transferred them to new MS plates containing 3% (w/v) glucose (Fig 4.3). The luminescence intensity from the primary screen was labeled on plates for secondary screens for comparison between each screen. The classified phenotypes of M<sub>2</sub> seedlings are given in the Table 4.1. These phenotypes include abnormal leaf size, senescence, dwarf, and giant phenotypes (Table 4.1 and Fig. 4.4). The luminescence intensity was also determined for each class of mutant.
Figure 4.2. Some EMS-mutagenized M₂ seedlings displayed developmental phenotypes including late germination, albino, dwarf, and lethality.

Images were taken from five-day-old seedlings grown on MS plates with different concentrations of glucose.
Figure 4.3. Mutant screen procedure.

About 500 seedlings were grown in 3% (w/v) glucose plates (left panel) and were screened with LUC image system. The primary screened putative mutants were rescreened on MS plates with 3% glucose (right panel). The color bar on the right shows luminescence intensity from lowest (black/white) to highest (red).
<table>
<thead>
<tr>
<th>Phenotypes</th>
<th>Numbers of mutants (N=39)</th>
<th>Relative luminescence intensities (RLU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT (#244)†</td>
<td>250</td>
<td>&lt; 250</td>
</tr>
<tr>
<td>No morphological phenotype</td>
<td>14</td>
<td>770-12,000</td>
</tr>
<tr>
<td>Large leaves</td>
<td>5</td>
<td>1,090-5,100</td>
</tr>
<tr>
<td>Early senescence</td>
<td>2</td>
<td>580-1,000</td>
</tr>
<tr>
<td>Dwarf</td>
<td>5</td>
<td>910-3,320</td>
</tr>
<tr>
<td>Tall</td>
<td>4</td>
<td>1,500-5,090</td>
</tr>
<tr>
<td>Small leaves and bushy (not dwarf)</td>
<td>2</td>
<td>1,130-1,500</td>
</tr>
<tr>
<td>Lethal</td>
<td>7</td>
<td>1,000-5,470</td>
</tr>
</tbody>
</table>

Table 4.1. Developmental phenotypes of putative mutants.

Luminescence intensities were measured on 7 d old seedlings in 3% glucose plates using Argus-20 system.

§ Numbers are from the screens on 4,8000 seedlings that survived on kanamycin plate (50µg ml⁻¹).

† A highest value from the measurements of luminescence intensities of 250 WT (#244) seedlings which grown in the same condition as the mutant screens.
Figure 4.4. Examples of selected mutants.

Three-week-old putative mutants and WT (Col-0) grown in the growth chamber under long-day (16h light/ 8h dark) conditions at 21°C.

Scale bars = 1cm.
4.5 DISCUSSION

Many *Arabidopsis* sugar-response mutants have been identified in screens employing the conditions that caused plant developmental arrest by applying high concentrations (e.g. 6%) of exogenous sugars. Several different groups have also used reporter-based screening protocols. I have developed a screen using an extremely sugar sensitive promoter *ASN1* and lower concentrations of sugars (discussed in chapter 2). Bioluminescence reporter luciferase (LUC) has a high turnover rate which can be used to monitor the change of gene expression (Millar et al., 1992) and the LUC assay has low background bioluminescence that can distinguish subtle difference between mutant and WT plants (Yamamoto et al., 2003). In addition, the luciferase imaging system is very efficient for the isolation of mutants in high throughput fashion (Xiong et al., 1999). In a single Petri-dish plate, I planted 500 mutagenized *Arabidopsis* seeds and processed an image analysis in 5 min. With this system, I could screen over 8,000 seedlings in 2 h.

About 50 putative mutants have been identified from 8,0000 EMS-mutagenized seedlings. They have pleiotropic developmental abnormalities including abnormal seedling and leaf phenotypes, dwarfism, delayed flowering, and no apical dominance. It is yet to be determined whether the mutants contain either additional mutations important for growth and development or a single mutation involved in both sugar signaling and developmental regulation (Table 4.1). The putative mutants showed pleiotropic phenotypes with similar levels of luminescence, implicated that mutants might have lesions at multiple loci. In addition, no direct connection between phenotype and
luminescence levels, suggested that different sugar response genes could be involved in these mutants. The putative mutants showing early senescence phenotype expressed relatively low intensities of luminescence probably caused by the fact that they had lower biomass. It is possible that the pleiotropic features are due to the effects of the mutations on sugar-regulated genes that play important roles in growth and development. The effects of these mutations on the expression of \textit{ASN1} could be an indirect effect of an altered growth response of mutant plants to sugars.

As \textit{ASN1} is preferentially expressed in sink tissues, mutants with abnormal sink to source transition might potentially be isolated as sugar signaling mutants. For instance, a number of putative mutants were giant in size (Fig. 4.3A), with numerous rosette leaves before bolting. Alternatively, individuals with altered circadian regulation might also be identified as sugar signaling mutants, because \textit{ASN1} is also regulated by the clock (Harmer et al., 2000). This could be verified by examining the luminescence in a time course experiment in the future.

This novel genetic screen was designed to avoid the use of high sugar media, therefore, less likely to suffer from osmotic side effects. Although I expect these sugar-response mutants to be more directly involved in sugar signaling, but still it is highly possible to isolate mutants involved in ABA sensing and signaling. Therefore, further manipulation of the screen conditions, such as reducing the glucose concentration down to 1 % (w/v) could reduce the chance of getting stress-related mutants. Alternatively, mutants could be screened by higher resolution image analysis system. For example, increasing sensitivity can be attained by using a liquid nitrogen-cooled CCD camera since the cryogenic cooled CCD camera systems will often give higher spatially resolved
images than the intensified CCD systems because the resolution is degraded in the microchannel plate of the intensified camera (Kay et al., 1994). In addition, a luminometer could be used to screen seedlings grown in microtiter plates on MS medium with low levels of sugars as demonstrated in the previous mutant screen (Baier et al., 2004).

4.6 FUTURE PERSPECTIVES

Future molecular, genetic, and phenotypic analysis of putative mutants

I expect that some mutants may contain intragenic mutation occurring within the marker gene. I also expect that extragenic mutation (trans) occurs outside of the genetic code, but has an effect on the marker gene. These mutations can be determined by comparing the endogenous and transgene expression. Whereas transgene expression is abnormal in intragenic mutants, their endogenous ASN1 expression should be normal. In contrast, both the endogenous and the transgene expression are expected to be abnormal in extragenic mutants. To determine whether mutation is dominant or recessive, I will backcross the putative mutants to the parental P\textsuperscript{ASN1::LUC} marker line. F\textsubscript{1} and F\textsubscript{2} generations will be scored for phenotype and LUC activity. If the mutation is recessive, all F\textsubscript{1}-plants should show wild-type phenotype. If the mutation is dominant, there are two possibilities: if the original mutant was homozygous, all F\textsubscript{1}-plants will show the mutant phenotype. If original mutant was heterozygous, the progeny should segregate 1:1 (wild-
type: mutant). In F\textsubscript{2} progeny from self-pollination of F\textsubscript{1}, if the original mutant was dominant and homozygous genotypes, the segregation will be 1:3 (wild-type: mutant). If the original mutant was recessive and homozygous genotypes, the segregation will be 3:1 (wild-type: mutant). When individual mutants are mapped, mutant complementation analysis will be performed by pair-wise crossing of the mutants that are mapped to the same locus. Mutants allelic to each other are expected to have a non-complementary phenotype.

Glucose has been implicated to be the primary sugar signal that controls many aspects of plant development from seed germination to senescence. I found that high levels of glucose specifically inhibited seed germination, hypocotyl elongation, root development, and greening of cotyledons. I will examine the mutants to see whether they display any abnormal responses to high levels of glucose during development. If they do, the mutation is expected to be involved in a step that is very upstream of the sugar-signaling pathway, as both \textit{ASN1} expression and developmental processes are affected. I would assume that the mutation is involved in a pathway or step that is specific to the control of \textit{ASN1} expression if the mutants have no developmental phenotypes. Results of our recent DNA microarray analysis indicated that many metabolic, structural, and regulatory genes are rapidly up-regulated or down-regulated by glucose (Price et al., 2004). Because global gene expression profiling is very efficient, I will perform DNA microarray analysis for at least one mutant in each complementation group. The results are expected to be a powerful tool for the prediction of the function of mutated genes. Mutants will be mapped and mutation locus will be cloned using standard protocols (Konieczny and Ausubel, 1993; Bell and Ecker, 1994; Lukowitz et al., 2000).
Comparison between WT plant and the mutant will yield information about genetic lesion and/or action of gene products. The subcloned DNA will be transformed into *Arabidopsis*, looking for complementation of the mutation among the transformants. I will overexpress the targeted gene in the WT background to determine gain-of-function phenotype.

Among the glucose-insensitive mutants, some of them may not be caused by mutation in glucose signaling pathways. These include mutants defective in light signaling, HXK, sugar metabolism, or sugar transporter. To avoid obtaining mutants involved in secondary processes described above, I will verify the mutants using a short term (<3 h) and lower level of glucose (<3%). Furthermore, some of the non-specific mutants can be verified by performing various molecular or phenotypic assays. The glucose uptake assay will be performed to examine whether mutants have any defects in glucose uptake by using C$^{14}$-labeled glucose or other sugar analogs (Zhou et al., 1998). I will compare the uptake rates of glucose by using seedlings or protoplasts of both mutants and wild-types. Finally, light signaling mutants can be verified by examining the endogenous *ASN1* and transgene expression in response to glucose in the dark.

When the study is completed, I expect to have identified mutants that are specifically involved in glucose signaling in plants. Cloning of the gene mutated in each mutant may lead to identifying new glucose signaling components. These components will be characterized using biochemical, cellular, and molecular methods. As *ASN1* is involved in nitrogen metabolism, the understanding of this pathway has direct impact in plant growth, development, and senescence controlled by C/N balancing.
Knowledge of the molecular basis of gene structure and functions that regulate mechanisms that control plant carbon and nitrogen nutrition will provide opportunities to engineer new useful traits in crop plants. Results of this research are expected to advance our understanding in basic plant biology as well as to provide a fundamental mechanism for crop improvement. I would use gene knockout technology if reducing the expression of a discovered gene would enhance crop productivity. Conversely, I would use the overexpression technique if the crop yield were correlated with the level of gene expression.
5.1 ABSTRACT

In bacteria, ACT domains have been shown to serve as amino acid-binding sites for feedback regulation of metabolic enzymes involving in nitrogen metabolism. From the recent DNA microarray analysis (Price et al., 2004), I have found that the transcription of two ACT domain-containing protein kinase genes are highly regulated by glucose. In the current study, I propose that the ACTPK may function as a regulator of C/N sensing in plants. Toward testing this hypothesis, Northern blot analysis has been conducted to determine sugar response, the temporal and spatial expression of ACTPK3. I have found
that the *ACTPK3* transcript level was completely abolished by low levels of exogenous glucose (0.1%, w/v) in 3 h. Interestingly, *ACTPK3* is likely regulated by the circadian clock that is epistatic of response triggered by exogenous sugar. While glucose and mannose, both are substrates of HXK, repressed *ASNI* expression, a non-substrate of HXK 3-*O*-methyl-glucose and a non-transportable sugar L-glucose did not cause any repression on *ACTPK3*. These results implicate that *ACTPK3* repression requires sugar transport, phosphorylation, and HXK. I have also found that *de novo* protein synthesis is required for both sugar and clock regulation of *ACTPK3* by treating the plants with cycloheximide (CHX), a general protein synthesis inhibitor. The repression of *ACTPK3* was inhibited by the protein phosphatase inhibitor okadaic acid, but not by the serine/threonine protein kinase inhibitor, K-252a, suggesting a requirement for protein dephosphorylation in *ACTPK3* repression. I have further tested whether this glucose repression was independent of ABA. Results showed that the repression of *ACTPK3* occurred even in the presence of fluridone, an ABA biosynthesis inhibitor. Although ABA could enhance *ACTPK3* expression at a low level (0.1µM), glucose induced *ACTPK3* repression persisted in the presence of exogenous ABA. Therefore, the *ACTPK3* repression by glucose was not mediated through ABA under our experimental conditions. In addition, T-DNA insertion mutants in *ACTPK* genes have been obtained from ABRC. The phenotypic analysis revealed that the mutants are hypersensitive to low levels of glucose. Based on the analyses of putative loss-of-function mutants, I concluded that the *ACTPK* could be involved in sugar, nitrogen, and/or C/N sensing in plants.
Development and metabolism in plants are closely linked at various levels. Although underlying molecular mechanisms are still not fully understood, overall metabolic coordination in plants is likely mediated through global regulatory mechanisms that sense and respond to the changes of C/N metabolites. Recent studies have begun to reveal components involved in C/N sensing and signaling mechanisms in plants. Compared with unicellular organisms, such as *E. coli* or yeast, plants have evolved to use more complex C/N sensing and signaling systems since different developmental stages and tissues may require specific levels of C and N (Coruzzi and Zhou, 2001).

Some plants can support bacteria that convert inorganic nitrogen into ammonia by fixing atmospheric dinitrogen into ammonia in their symbiotic root nodules (e.g. *Rhizobium* and *Frankia*). As the primary source of nitrogen in plants, nitrate is reduced to nitrite in the cytoplasm by nitrate reductase and then transported into the chloroplasts. In the chloroplasts, the nitrite is further reduced to ammonium. Two enzymes, the Gln synthetase (GS) and Glu synthase (GOGAT) are involved in assimilation of ammonium into primary amino acid synthesis in plants. In the GS/GOGAT cycle, the coordination of carbon (C) metabolism and nitrogen (N) metabolism is required since GS requires C skeletons in the form of 2-oxogluarate and reductant in the form of ferredoxin or NADH (Lancien et al., 2000). The supplementation of growth media with sucrose and organic
nitrogen can cause changes in the expression of some nitrogen assimilatory genes, such as those encoding the enzymes nitrate reductase (NR) (Cheng et al., 1992; Faure et al., 1994), glutamine synthetase (GLN) (Vincentz et al., 1993; Lam et al., 1995), and asparagine synthetase (ASN) (Lam et al., 1995). In the bacteria, the PII signal transduction protein plays an important role in sensing and coordination for C and N metabolism (Magasanik, 2000; Ninfa and Atkinson, 2000). PII was first recognized when it was discovered that the addition of ammonia to a culture of E. coli growing on a poor source of nitrogen resulted in the inactivation of GS due to its adenylylation by the enzyme adenylyl transferase (ATase), and that ATase was also capable of catalysing the deadenylylation of GS–AMP which results in the inactivation of GS. In another case, when Gln levels are low and both C and energy status is adequate, PII is uridylylated by the UT/UR. Then, PII-UMP stimulated the activation of GS. In the absence of affinity between NRII and PII, it allowed NRII to activate NRI and the promoter transcription of the N-sensitive regulation. In plants, PII homolog GLB1 has been found in more than 20 species including castor bean, alfalfa, soybean, tomato, rice, and Arabidopsis (Moorhead and Smith, 2003). In Arabidopsis, PII gene was regulated by light and metabolites. In addition, the organic nitrogen Gln was much less effective in reversing the sucrose-induced anthocyanin accumulation in PII overexpressors than WT, suggesting that GLB1 is involved in sensing the status of C and organic N in higher plants (Hsieh et al., 1998).

Recent DNA microarray analyses in our lab (Price et al., 2004) have shown that genes involved in nitrogen metabolism are largely regulated transcriptionally by sugar rather than inorganic nitrogen, thus these results explained the significance of the role of carbon/nitrogen ratio in plant growth and development. Among those nitrogen metabolic
genes, two putative ACT domain-containing protein kinase genes are highly regulated by glucose. The ACT domain, amino acid binding domain was named after aspartokinase, chorismate mutase, and TyrA (Aravind and Koonin, 1999). The ACT domains have been shown to serve as amino acid-binding sites for feedback regulation of metabolic enzymes involved in amino acid and purine metabolism in bacteria (Schuller et al., 1995; Aravind and Koonin, 1999; Chipman and Shaanan, 2001). Based on the database search (Pafm, http://www.sanger.ac.uk), I found that the ACT domain-containing genes were nearly ubiquitous from the prokaryotes to the eukaryotes. In Arabidopsis, eight ACR genes were previously found by searching the database and they showed high amino acid sequence similarities among ACR family (Hsieh and Goodman, 2002). These ACR genes had four repeats of ACT domain. The molecular analysis on the ACR genes showed that these genes were differentially regulated by hormones, salt, or cold stress, suggesting that ACR proteins could be involved in the networks of metabolic or hormonal signaling pathways in plants (Hsieh and Goodman, 2002). In ACT domain-containing proteins, the regulatory ACT domain is usually linked to a metabolic enzyme (Aravind and Koonin, 1999). Similarly, in Arabidopsis, ACT domain is associated with functionally diverse enzymes (Table 5.1) including the amino acid metabolic enzyme Asp kinase (AK), AK/homo-Ser dehydrogenase (AK/HSD), phosphoglycerate dehydrogenase (PGDH), prephenate dehydratase (PDH), protein kinase (PK), ACR gene family, and acetolactase synthetase (ALS). In current research, from the database analysis, I have found 27 ACT domain-containing genes in Arabidopsis and three of those were ACT domain-containing protein kinases (ACTPK1, -PK2, and –PK3). Differential regulations of ACTPK3 with various growth conditions, sugars, nitrogen, and hormones will be described.
5.3 MATERIALS AND METHODS

Plant materials

T₄ seeds of Salk T-DNA lines (http://signal.salk.edu/tabout.html) were obtained from the Arabidopsis Biological Resource Center (ABRC, The Ohio State University). Arabidopsis wild-type and T-DNA lines were grown in the growth chamber under 16 h/8h light/dark cycle or on MS plates under constant white light (90 μE m⁻² sec⁻¹). Wild-type and transgenic Arabidopsis plants were the Columbia-0 ecotype unless otherwise specified. Seed batches were grown simultaneously, harvested, and stored for at least a month before using them in the experiments. For in vitro growth, a liquid culture system (Cheng et al., 1992) was employed in the experiments for gene expression analysis. Seeds were surface-sterilized in 95% (v/v) ethanol for 1 min followed by 50% (v/v) commercial bleach (Clorox) for 15-20 min, and rinsed five times with sterile water. After a 3 d cold treatment at 4°C in the dark, approximately 1,000 seeds were transferred into 125 ml Erlenmeyer flasks containing 20 ml 1x basal Murashige and Skoog media (MS) (Murashige and Skoog, 1962) and left at 4°C for another 4 d complete stratification. Plants were grown on a rotary shaker (New Brunswick Scientific, Edison, NJ) with constant agitation at 128 rpm under constant light (90 μmol m⁻² s⁻¹) at 22°C for 6 d. Seedlings were then transferred to the dark for 1 d in a sugar-free medium to deplete
endogenous sugars. Plants were collected for RNA extraction at the end of the treatments.

**Treatments with chemical antagonists**

Cycloheximide was obtained from Sigma-Aldrich (St. Louis, MO) and dissolved in distilled water at 10mM. Okadaic acid and K-252a were purchased from A.G. Scientific, Inc. (San Diego, CA) and dissolved in dimethyl sulfoxide (DMSO) at 1mM to yield stock solution. After the addition of the selected reagents, the flasks were returned to the original culture conditions. For controls, seedlings were treated with equivalent amounts of distilled water or DMSO; these treatments did not affect transcript levels.

**RNA Isolation, RT-PCR, and Northern Blot Analysis**

From ground plant material which was frozen with liquid nitrogen, RNA was extracted by using either RNeasy® Plant Mini Kit (Qiagen, Hilden, Germany) according to the modified manufacturer’s protocol or our laboratory protocol (Xiao et al., 2000). All experiments were repeated at least twice with RT-PCR or Northern blot analysis. RT and control reactions were performed described in the manufacture’s protocol (Takara One-Step RNA PCR kit, Madison, WI). The cDNA samples and RNA amounts were standardized with analysis of spectrophotometer and ethidium bromide gel. For Northern blot analysis, 5 µg of each total RNA sample was separated on 1.2% (w/v) agarose gels and subsequently blotted onto a nylon membrane (ICN, Irvine, CA), using the standard procedures (Sambrook *et al.*, 1989). RNA was fixed to the blot using UV crosslinker (Invitrogen, Carlsbad, CA) with 0.120 J and subsequently baked at 80°C for 1h.
Radiolabeled DNA probes were prepared by PCR reactions with a 20 µl mixture containing 1x labeling buffer, 0.2 mM deoxynucleotide triphosphate (without ATP), 0.25 units of Taq polymerase (New England Biolab, Beverly, MA), 0.05 µg primers, 0.1 µg template DNA, and 1 µCi 32P-α-ATP. Forty cycles of PCR amplification were carried out at 94ºC for 30 sec (denaturation), 52ºC for 45 sec (annealing), and 72ºC for 60 sec (synthesis). The gene-specific PCR primers are as follows: ACTPK3 (GenBank accession no. NM_120008), forward, 5’- GACTAGT ATGGTGATGG AGGACAA -3’; reverse, 5’- CAT GCC ATG GGA TGT GTG GTG CTT CTC. A plasmid, ACTPK3::GFP/GUS was used as template DNA for PCR. The radiolabeled probe was separated from the unincorporated dNTPs by chromatography through a spin column of Sephadex G-50. The purified radiolabeled cDNA was used for hybridization with denaturation by heating for 5 min at 100ºC and chilling the probe rapidly in ice water. Radiolabeled cDNA activity of 1.5 x 10^6 cpm ml^-1 was used for each membrane. That membrane was hybridized for 12-16 hours in a rotating hybridization oven (Robbins Scientific, Sunnyvale, CA) and washed with 500 ml of 2x SSC containing 1% (w/v) SDS for 15 min at room temperature, then washed 3 times with 0.5x SSC containing 1% (w/v) SDS for 30 min at 60ºC, then subjected to autoradiography.

Sequence Analysis

The ACT domain-containing protein kinase genes (ACTPK) in Arabidopsis were verified by searching public databases at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/) and the Arabidopsis Information Resource (http://www.Arabidopsis.org/Blast/). The ACTPK sequences were aligned using
the ClustalW 1.8 program (http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html) to determine sequence identity and similarity among the *ACTPK* genes. For analysis of the phylogenetic relationship among the ACT domain-containing protein kinases, various proteins were aligned to generate the phylogenetic tree using the QuickTree with A-Tree-viewer (ATV) (http://www.sanger.ac.uk/Software/analysis/quicktree/) (Zmasek and Eddy, 2001; Howe et al., 2002).

**Genomic DNA extraction**

Genomic DNA was extracted with the modified method described by the Wisconsin *Arabidopsis* Knockout Facility (Krysan et al., 1999). Plants were grown in a sugar-free medium for 18 d under constant light. Leaf tissues (3-4 leaves) were ground in a 500 µl extraction buffer (0.2 M Tris-HCl, pH 9.0, 0.4 M LiCl, 25mM EDTA, and 1% SDS) in a microcentrifuge tube. After spinning down for 5 min at 12,000 rpm in a microcentrifuge, the 350 µl of supernatant was transferred to a fresh microcentrifuge containing 350 µl isopropanol. It was mixed by inversion and spun 10 min at 12,000 rpm in a microfuge. Pellets were dried and resuspended in 400 µl TE.

**Homozygous T-DNA knockout mutants screens**

Gene specific primers were designed using a primer design program (http://signal.salk.edu/tdnaprimers.html). The gene-specific primers were: SALK_065422 LP: CCCTGGTTTCGAAGACGACCT, RP: CCCAATTCTTCCAAGCAACAAG and SALK_116340 LP: CGGAAAAGGAAAATGGGCAA, RP:
TCCTGTATAACGCAAGCTCAAGGA. The reaction mixture (final volume 25µl) consisted of 2.5 µl 10X reaction buffer, 1.0µl 2.5mM dNTPs, 2.0µl genomic DNA (isolated by the methods described above), 0.5µl primer RP (0.5 µg µl⁻¹), 0.5µl primer LP (0.5 µg µl⁻¹), 0.5µl Taq polymerase (5U µl⁻¹, NEB, Beverly, MA), and distilled water to bring the final volume to 25µl. The following program was used for the PCR reactions: 40 cycles of 94 °C for 30 sec, 62 °C for 45 sec, and 72 °C for 1min.

Phenotypic analysis of sugar sensitivity in T-DNA insertion lines

T-DNA lines from SALK (Alonso et al., 2003) (SALK_116340-6 and SALK_065422-1) and the wild-type Colombia were germinated and grown on MS medium with 3% (w/v) glucose or without sugar. Plates were set vertically and the seeds were allowed to germinate and grow in constant white light (90 µE m⁻² sec⁻¹). Images were recorded using a digital camera (Canon, Lake Success, NY) on the 5th and 18th day.

5.4 RESULTS AND DISCUSSION

5.4.1 ACTPK family in Arabidopsis

In searching the protein database (Pfam, http://www.sanger.ac.uk/Software/Pfam/) (Bateman et al., 2004), more than 1,000 ACT domain containing genes were found from bacteria to eukaryotes including humans and plants. In plants, ACT domain proteins were found in six different species: Arabidopsis
thaliana, soybean (Glycine max), carrot (Daucus carota), tobacco (Nicotiana plumbaginifolia), maize (Zea mays), and rice (Oryza sativa). Except for Arabidopsis and rice, only a few proteins were detected in other plant species, in which complete genome information has not been available. I have found 27 putative ACT domain-containing genes in the Arabidopsis genome (Table 5.1). The 27 ACT domain-containing proteins were divided into eight groups based on the predicted functional domain(s) in the protein sequence (Table 5.1). These groups consisted of previously reported ACT domain containing proteins (Aravind and Koonin, 1999). However some ACT domain proteins such as chorismate mutases, malate dehydrogenases, phenylalanine and tryptophan-4-monooxygenases, phosphoribosylformyl glycaminidyl synthase, GTP pyrophosphokinase/phosphohydrolase(SpoT/RelA), and tyrosine and phenol metabolism operon regulators (TyrR) were not reported in Arabidopsis. Among the 28 genes, I also found eight ACR genes which were reported previously (Hsieh and Goodman, 2002). The ACR3 gene was preferentially expressed in the source tissue, suggesting a possible role in source-sink transition in plant cells (Hsieh and Goodman, 2002).

Four ACTPK genes were detected in plants: three in Arabidopsis and one in rice. Although most ACT domain containing genes were not experimentally characterized, four Arabidopsis ACTPK were predicted (Hsieh and Goodman, 2002). However, I found two genes to be identical by blast search (http://www.ncbi.nlm.nih.gov/BLAST/). The function of the three ACTPK has not been reported to date. I named them as: ACTPK1 for At2g17700 (GenBank accession no. NP_179361); ACTPK2 for At4g35780 (GenBank accession no. NP_195303); ACTPK3 for At4g38470 (GenBank accession no. NP_568041).
The phylogenetic tree containing these four genes from *Arabidopsis* and rice are shown in Fig. 5.1, which indicates that the ACT domain gene family members have evolved and diverged into a distinct subgroup. The *ACTPK1, ACTPK2*, and rice *ACTPK* are closely related. The *ACTPK3* belongs to a different but also closely related clade. It is interesting that *ACTPKs* are more closely related to GlnD in bacteria than to other ACT domain-containing genes (Fig. 5.1). In *E. coli* sensor protein, GlnD is composed of a nucleotide transferase domain, a nucleotide hydrolase domain, and two C-terminal ACT domains. In addition, GlnD encoding uridylyltransferase (UT)/uridylyl removing (UR) enzyme, which acts as the primary nitrogen sensor in the nitrogen regulation (Ntr) system (Arcondeguy et al., 1997; Hsieh et al., 1998; Ninfa and Atkinson, 2000). These three *ACTPK3* have high identities to each other: *ACTPK1* and *ACTPK2* share 79% amino acid and 68% nucleotide sequence identity throughout their entire length. The *ACTPK1* and *ACTPK3* share 62% amino acid and 58% nucleotide sequence identity. The *ACTPK1* and *ACTPK3* share 60% amino acid and 58% nucleotide sequence identity (Table 5.2).

The results of our DNA microarray analysis showed that among these *ACTPK* genes, *ACTPK2* and *ACTPK3* were repressed by exogenous glucose by 12.5- and 3.5-fold, compared respectively with the non-treated control (Table 5.3). While the treatment with the combination of C (167 mM exogenous glucose) and N (40 mM nitrate plus 20 mM ammonium) somewhat relieved the C suppression, the N source without C did not induce the expression of *ACTPK3*. With these results, I hypothesized that *ACTPK* could be involved in C/N sensing in *Arabidopsis*. 
<table>
<thead>
<tr>
<th>ACT domain-containing protein</th>
<th>AGI gene code</th>
<th>No. of ACT domain</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aspartate kinase (AK)</em></td>
<td>At5g13280, At5g14060, At1g76450</td>
<td>2</td>
</tr>
<tr>
<td><em>Putative aspartate kinase-homoserine dehydrogenase (AK/HSD)</em></td>
<td>At1g31230, At1g31230, At4g19710</td>
<td>2</td>
</tr>
<tr>
<td><em>Phosphoglycerate dehydrogenase-like protein (PGDH)</em></td>
<td>At3g19480, At4g34200, At1g17740</td>
<td>1</td>
</tr>
<tr>
<td><em>Putative prephenate dehydratase (PDH)</em></td>
<td>At1g11790, At3g07630</td>
<td>1</td>
</tr>
<tr>
<td><em>Protein kinase like protein (PK)</em></td>
<td>At2g17700 (<em>ACTPK1</em>)<em>, At4g35780 (<em>ACTPK2</em>)</em>, At4g38470 (<em>ACTPK3</em>)*</td>
<td>1</td>
</tr>
<tr>
<td><em>Putative acetolactate synthase (ALS)</em></td>
<td>At2g31810, At5g16290</td>
<td>2</td>
</tr>
<tr>
<td>ACR family (include putative uridylyl transferase (UTase))</td>
<td>At5g65890 (ACR1)<em>, At5g25320 (ACR2)</em>, At1g76990 (ACR3)<em>, At1g69040 (ACR4)</em>, At2g03730 (ACR5)<em>, At3g01990 (ACR6)</em>, At4g22780 (ACR7)<em>, At1g12420 (ACR8)</em></td>
<td>4</td>
</tr>
<tr>
<td>Other uncharacterized proteins</td>
<td>At2g39570 (3)<em>, At2g39570 (3)</em>, At5g04740 (1)*</td>
<td>1 or 3</td>
</tr>
</tbody>
</table>

Table 5.1. ACT domain-containing proteins in *Arabidopsis*.

† Number of ACT domains,

§ Gene name
Figure 5.1. Phylogenetic analysis of \textit{ACTPK} related proteins.

Number above each line indicates a branch length value, i.e. the number of changes that have occurred in that branch.
Table 5.2. Amino acid identities between the three genes in *Arabidopsis*.

<table>
<thead>
<tr>
<th>AGI gene code</th>
<th>At4g38470 (<em>ACTPK3</em>)</th>
<th>At4g35780 (<em>ACTPK2</em>)</th>
</tr>
</thead>
<tbody>
<tr>
<td>At4g35780 (<em>ACTPK2</em>)</td>
<td>60% (58%) †</td>
<td></td>
</tr>
<tr>
<td>At2g17700 (<em>ACTPK1</em>)</td>
<td>62% (58%)</td>
<td>79% (68%)</td>
</tr>
</tbody>
</table>

† Nucleotide identity.
<table>
<thead>
<tr>
<th>ID</th>
<th>AGI gene code</th>
<th>Control</th>
<th>C</th>
<th>N</th>
<th>C+N</th>
<th>3-OMG</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTPK1</td>
<td>At2g17700</td>
<td>1(176)</td>
<td>1.5(259)</td>
<td>1(181)</td>
<td>1.1(197)</td>
<td>1.3(220)</td>
</tr>
<tr>
<td>ACTPK2</td>
<td>At4g35780</td>
<td>1(526)</td>
<td>-3.2(164)</td>
<td>-1.1(502)</td>
<td>-3.5(144)</td>
<td>-1.1(456)</td>
</tr>
<tr>
<td>ACTKP3</td>
<td>At4g38470</td>
<td>1(2054)</td>
<td>-12.5(141)</td>
<td>1.2(2497)</td>
<td>-7.5(247)</td>
<td>-1.7(1487)</td>
</tr>
</tbody>
</table>

Table 5.3. The effects of carbon (C, 167mM glucose), inorganic nitrogen (N, 40mM nitrate and 20mM ammonium), and 3-OMG (167 mM, 3-O-methyl glucose) on the expression of three putative ACTPK genes in Arabidopsis.

Detailed plant materials preparation and GeneChip analysis procedure are given in the paper (Price et al., 2004). Fold changes were calculated by comparing the expression change between control (set as 1 fold) and treated samples. The numbers in the parentheses indicate raw expression values from the microarray analysis.
5.4.2 Factor affecting steady state transcriptional level of ACTPK3

5.4.2.1 Effects of light and glucose

The expression of ACTPK3 was examined by Northern-blot analysis using total RNA from WT (Col-0) Arabidopsis. Transcripts of ACTPK3 were suppressed by light with 3% (w/v) glucose in 1 h and were completely abolished in 3 h (Fig. 5.2). However, after 24 h of incubation with glucose, its expression started to peak again, indicating a potential circadian-clock regulation. A single light/dark (LD) or dark/light (DL) cue has been proven to be sufficient to generate a robust free-running rhythm (Nakamichi et al., 2004). With a period of approximately 24 h, the amounts of ACTPK3 transcripts were greater in plants transferred from continuous light (LL) to dark/light (DL) (Fig. 5.2) than plants transferred continuous light (LL) to continuous darkness (DD) (Fig. 5.3). In addition, under the constant light conditions (24h, sugar-free medium), the transcript level showed its highest levels, indicating light has additional effects on the induction of ACTPK3 transcripts. Circadian rhythms are fundamental for living cells to adapt to daily and seasonal light, and temperature fluctuation. Many genes involved in carbon and nitrogen metabolism are regulated by the circadian clock (Harmer et al., 2000). In mammals, restricted feeding also entrain behavioral and physical circadian rhythms, which depend on a food-entrainable oscillator (Ben-Shlomo and Kyriacou, 2002). In plants, no studies have been performed to investigate the relationships between nutrient signaling and circadian clock regulation. However, further experiments are required for verifying whether ACTPK3 is affected by circadian regulation.
To test the dose effect of glucose on the expression of *ACTPK3*, up to 5% (w/v) glucose was applied to 7 d seedlings. Exogenous 0.1% (w/v) glucose was sufficient to suppress *ACTPK3* transcript levels in 3 h (Fig 5.3). Intriguingly, although its expression level was low, the supplement of 5 % exogenous glucose caused less expression of *ACTPK3* mRNA.

**5.4.2.2 The role of ABA in glucose repression of *ACTPK3***

It was not clear whether the repression of the *ACTPK3* with higher levels of glucose (5%, w/v) (Figure 5.3) could be mediated through ABA. It was previously demonstrated that cellular ABA concentration increased when plants were treated with high concentrations of exogenous glucose (Price et al., 2003) (also showed on Figure 2.1 in Chapter 2). To investigate the role of ABA in glucose repression of *ACTPK3*, a series of RNA gel blot analyses was conducted. Interestingly, I found that low levels (0.1 µM) of ABA enhanced *ACTPK3* expression but as the amounts increased, the expression levels decreased (Fig. 5.4). The previous experiment using *ASN1* also showed similar patterns of transcriptional expression with different amounts of ABA (Fig 2.11 in Chapter 2). It may be possible that high amounts of ABA could exert stress effects which may degrade or destabilize transcripts in these genes. Nevertheless, these ABA inductions were abolished by exogenous glucose (Fig. 5.4). The addition of the ABA biosynthesis inhibitor, fluridone did not change glucose repression of *ACTPK3* (Fig. 5.4). These results implicated that phytohormones ABA were not involved in the repression of *ACTPK3* by glucose.
Figure 5.2. Kinetics of light and glucose repression of \textit{ACTPK3}.

RNA gel blot analysis was used to determine the steady state mRNA level of \textit{ACTPK3}. The WT (Col-0) plants were grown in MS liquid medium with 3% glucose in continuous light for 6 days. Plants were then washed 3 times with sugar and nitrogen free MS medium and dark adapted for 24 h to deplete internal sugar and nitrogen. Plants were then treated with 3% glucose in the light for various amount of time before tissue collection and RNA extraction. Five micrograms of total RNA was loaded in each lane. Equal loading is indicated by uniform intensity of rRNA bands stained by ethidium bromide. MS 3 h: RNA sample collected from identically prepared plants except that they were incubated in sugar-free MS in the dark for 3 h in the last step. 0: RNA sample collected from plants after the dark adaptation. Continuous light: RNA collected from plants grown in MS with 3% glucose in continuous light for 8 days. LL: continuous light. D: dark. L: light.
RNA gel blot analysis was used to determine the steady state mRNA level of ACTPK3. The WT (Col-0) plants were grown in MS liquid medium with 3% glucose in continuous light for 6 days. Plants were then washed 3 times with sugar and nitrogen free MS medium and dark adapted for 24 h to deplete internal sugar and nitrogen. Plants were then treated either with 3% glucose in the dark for various amount of time (time-course experiment) or with various concentrations of glucose in the dark (glucose dosage effect) before tissue collection and RNA extraction. Five micrograms of total RNA was loaded in each lane. Equal loading is indicated by uniform intensity of rRNA bands stained by ethidium bromide. **MS 3 h**: RNA sample collected from identically prepared plants except that they were incubated in sugar-free MS in the dark for 3 h in the last step. **0**: RNA sample collected from plants after the dark adaptation. **LL**: continuous light. **DD**: continuous darkness.
Figure 5.4. Neither exogenous ABA nor inhibitor of ABA biosynthesis could block glucose repression of *ACTPK3*.

RNA gel blot analysis was used to determine the steady state mRNA level of *ACTPK3*. The WT (Col-0) plants were grown in MS liquid medium with 3% glucose in continuous light for 6 days. Plants were then washed 3 times with sugar and nitrogen free MS medium and dark adapted for 24 h to deplete internal sugar and nitrogen. Plants were then treated with glucose, ABA, and/or Fluridone in the dark for 3 to 12 h before tissue collection and RNA extraction. Five micrograms of total RNA was loaded in each lane. Equal loading is indicated by uniform intensity of rRNA bands stained by ethidium bromide. **MS 3 h**: RNA sample collected from identically prepared plants except that they were incubated in sugar-free MS in the dark for 3 h in the last step.
5.4.2.3 The role of hexokinase in glucose repression of ACTPK3

To investigate whether glucose-induced ACTPK3 repression involves sugar transport and phosphorylation, different sugars or glucose analogs were applied to the seedlings. Results showed that only glucose and mannose cause repression of ACTPK3 (Fig 5.5). In addition, neither 3-O-methyl-glucose (3-OMG) nor L-glucose could trigger repression of ACTPK3. This was unlikely due to osmotic effects since 3-OMG and mannitol did not repress ACTPK3 gene. Together these data suggest that ACTPK3 repression involves both sugar transport and phosphorylation and HXK may be required for ACTPK3 repression by glucose.

5.4.2.4 De novo protein synthesis is required for glucose repression of ACTPK3

To determine whether the repression of ACTPK3 required de novo protein synthesis, I examined the expression of ACTPK3 in the presence of the protein synthesis inhibitor cycloheximide (CHX). Results showed that the repression of ACTPK3 was partially blocked by both concentrations of CHX (50µM and 300µM) in 3 h (Fig 5.6), suggesting that de novo protein synthesis is required for the glucose repression of ACTPK3 in 3 h. I also analyzed our recent DNA microarray data which used similar experiment conditions as the Northern analysis used in this experiment except, in DNA microarray, 100 µM CHX was applied to seedlings 1 h before the addition of 3% (w/v) glucose. The DNA microarray results showed similar results as this Northern blot analysis, i.e. in the presence of CHX, ACTPK3 repression was relieved from –14.5 to –2.9 folds compared with the untreated control. The increased transcripts at 1h may have been caused by the stabilization of mRNA by CHX. The high doses (30-180µM) of the
protein synthesis inhibitors puromycin (PM) or CHX augment and stabilize mRNA transcript levels through mechanisms which are dependent on the complete inhibition of protein synthesis (Davidoff and Mendelow, 1994).

It is yet to be determined why glucose repression still occurred at 12 and 24 h time points and whether or not clock regulation of ACTPK3 expression is affected by CHX.

5.4.2.5 The role of posttranslational modification in glucose repression of ACTPK3

To understand whether glucose repression of ACTPK3 was mediated through posttranslational modification of protein, seedlings were treated with inhibitor of protein phosphatase type 2A, OKA, and Ser/Thr protein kinase inhibitor, K-252a. Results showed that glucose repression of ACTPK3 was blocked by the application of OKA, but not K-252a (Fig 5.7), suggesting that protein dephosphorylation is required for the repression of ACTPK3.

5.4.3 T-DNA knockout mutants of ACTPK3

To identify knockout mutants of ACTPK3, I searched the SALK sequence indexed insertion lines (Alonso et al., 2003) (http://signal.salk.edu/cgi-bin/tdnaexpress) and found two positive lines: SALK_065422 (GenBank accession no. BH813881) and SALK_116340 (GenBank accession no. BZ381158). The locations of T-DNA inserts in these lines in Arabidopsis genome are given in Figure. 5.8. Seeds obtained from the
Figure 5.5. *ACTPK3* repression involves sugar transport and phosphorylation.

RNA gel blot analysis was used to determine the steady state mRNA level of *ACTPK3*. The WT (Col-0) plants were grown in MS liquid medium with 3% glucose in continuous light for 6 days. Plants were then washed 3 times with sugar and nitrogen free MS medium and dark adapted for 24 h to deplete internal sugar and nitrogen. Plants were then treated with 0.1% sugar or glucose analogs in the dark for 3 h before tissue collection and RNA extraction. Five micrograms of total RNA was loaded in each lane. Equal loading is indicated by uniform intensity of rRNA bands stained by ethidium bromide. **MS 3 h**: RNA sample collected from identically prepared plants except that they were incubated in sugar-free MS in the dark for 3 h in the last step.
Figure 5.6. *De novo* protein synthesis is required transiently for glucose repression of *ACTPK3*.

RNA gel blot analysis was used to determine the steady state mRNA level of *ACTPK3*. The WT (Col-0) plants were grown in MS liquid medium with 3% glucose in continuous light for 6 days. Plants were then washed 3 times with sugar and nitrogen free MS medium and dark adapted for 24 h to deplete internal sugar and nitrogen. Plants were then treated with glucose and CHX for various amount of time in the dark before tissue collection and RNA extraction. Five micrograms of total RNA was loaded in each lane. Equal loading is indicated by uniform intensity of rRNA bands stained by ethidium bromide. **0**: RNA sample collected from plants after the dark adaptation. **MS 3 h**: RNA sample collected from identically prepared plants except that they were incubated in sugar-free MS in the dark for 3 h in the last step.
Figure 5.7. Protein dephosphorylation is required for the repression of *ACTPK3*.

RNA gel blot analysis was used to determine the steady state mRNA level of *ACTPK3*. The WT (Col-0) plants were grown in MS liquid medium with 3% glucose in continuous light for 6 days. Plants were then washed 3 times with sugar and nitrogen free MS medium and dark adapted for 24 h to deplete internal sugar and nitrogen. Plants were then treated with various concentrations of glucose and/or inhibitors for 3 h in the dark before tissue collection and RNA extraction. Five micrograms of total RNA was loaded in each lane. Equal loading is indicated by uniform intensity of rRNA bands stained by ethidium bromide.
ABRC were germinated on kanamycin selection media (50mg L\(^{-1}\)) and the individuals were tested by PCR to confirm the presence of the T-DNA insertion. Two gene-specific PCR primers (RP, right genomic primer and LP, left genomic primer) were used in combination with a primer specific to the T-DNA left border primer LBb1. PCR amplification using wild-type DNA yields a fragment of ca. 900 bp with the RP and LP primers, homozygous lines produce a band about 600 bp, and heterozygous lines generate both 900 bp and 600 bp bands. The results showed that SALK_116340 was a heterozygous line and SALK_065422 turned out to be a homozygous line. Homozygous progenies were obtained by allowing the heterozygous plants to self in the greenhouse.

To determine whether or not the putative T-DNA knockout plants had altered sugar sensitivity, phenotypic analysis was performed using 5-d-old seedlings grown on MS plates with 3% (w/v) glucose. Interestingly, the SALK_116340 line showed hypersensitivity to 3% (w/v) glucose. Compared with Col-0 line, SALK_116340 line showed inhibition of root growth (Fig. 5.9). Intriguingly, leaves of 18-day-old SALK_065422 appeared yellow-green. Whereas high glucose repressed chlorophyll accumulation in the wild-type plants, glucose insensitive2 (gin2) mutants remained green under the same culture conditions (Moore et al., 2003). It would be interesting to find out whether ACTPK3 plays any role in chlorophyll synthesis related to sugar signaling in plants. Results of this phenotypic analysis suggest that loss-of-function of ACTPK3 resulted in a sugar hypersensitive response (Fig. 5.10).
Figure 5.8. T-DNA insertion lines of ACTPK3. "|" indicate nearest insertion point of T-DNA and "|--==-->" indicate T-DNA/Tn. The detailed information is described in the text. The yellow colored nucleotides indicate exon region, purple indicates intron, and red colored types indicate UTR region. Blue highlighted type, ATG and TGA, indicate starting codon and stopping codon, respectively.
Figure 5.9. Phenotypic analyses of ACTPK3 lines.

**Upper panel:** T-DNA insertion lines and WT (Col-0) seedlings were grown on vertically MS plates with 3% glucose for 5 days. Scale bar = 1 mm between each line.

**Lower panel:** T-DNA insertion lines and WT seedlings were grown on MS plates without sugar for 18 days.
Figure 5.10. Proposed model for the role of ACTPK3 in plant sugar response.

The model illustrates that the mutant defected in the function of ACTPK3 gene could result in hyper-response to sugar (B).
5.5 CONCLUSION AND FUTURE PERSPECTIVES

The regulatory ACT domain, which fused to a variety of enzymes, enabled these enzymes to react to the respective ligands. For example, in *E.coli* 3-phosphoglycerate dehydrogenase (PGDH) C-terminal residue ACT domain was inhibited allosterically by L-serine (Grant et al., 1996) and the removal of the regulatory domain was sufficient to eliminate serine inhibition (Bell et al., 2002). In plants, the protein or regulatory domains involved in C/N metabolism is still unclear.

ACT domain exists in diverse metabolic enzymes in *Arabidopsis* such as AK, AK/HSD, PGDH, PDH, PK, and ACR (Fig. 5.1). Although feedback inhibition of amino acid metabolism via amino acid binding to the ACT domain has not been demonstrated in plants, a recent study indicates that this mechanism may be conserved in plants (Lee and Duggleby, 2001). Acetohydroxyacid synthase (AHAS) regulatory subunit contains two ACT domains. Leucine binds the first ACT domain and Valine or Isoleucine binds the second ACT domain repeat of AHAS in *Arabidopsis* (Lee and Duggleby, 2001). These results suggested that the conserved ACT domains might serve as a regulatory function in plants. In the current research, the results showed that transcripts of two *ACTPK* genes were regulated coordinately by glucose. In addition, the circadian-clock is likely involved in the regulation of *ACTPK3* expression. Interestingly, the putative loss-of-function mutants showed hypersensitivity to exogenous sugar. To further characterize the role of
ACT-PK genes in C/N sensing, I will characterize gain- of-function and loss-of-function mutants.

To better understand the function of Arabidopsis ACTPK3 proteins in C/N metabolism and signaling, it could be important to examine whether 1) the ACTPK activity is regulated by sugar or nitrogen; 2) ACTPK activity is involved in transmitting sugar or nitrogen signals; 3) loss- or gain-of-function of ACTPK will cause altered response to sugar, nitrogen, or both nutrients; 4) the relationship between ACTPK activity and nitrogen metabolism.
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