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IDENTIFYING THE *cis*-ACTING SUGAR RESPONSE ELEMENTS THAT FACILITATE GLUCOSE SIGNALING IN *Arabidopsis thaliana*

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Genetics

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by
Frank J. Barretta Jr.
December 2008

Accepted by:
Dr. Brandon Moore, Committee Chair
Dr. William Marcotte Jr.
Dr. Kerry Smith

ABSTRACT

Identifying the initial sugar sensing and response activating mechanisms in plants has been difficult due to the dual functions of sugars as nutrients and as signaling molecules. In this study, we have examined transcript expression and promoter features of different genes encoding proteins for actin remodeling that have been implicated as targets of plant glucose signaling. Using Reverse Transcription PCR analysis, we confirmed that expression of two actin-associated genes, actin depolymerizing factor 9 (ADF9) and actin related protein 8 (ARP8), are repressed following a short-term glucose treatment of *Arabidopsis thaliana* seedlings. Glucose did not repress the expression of other ADF and ARP gene family members. The *cis*-acting promoter elements of both gene families were evaluated using the PLACE database. This analysis indicated that the ARP8 promoter has a unique signature motif, a four amylase box 1 repeat, that might account for the observed glucose dependent repression response. A unique response element motif was not readily identified in the predicted promoter region of ADF9. To further define possible sugar response elements, the predicted promoters of ADF9 and ARP8 were cloned as luciferase fusions in a plant expression vector. However, by transient expression assay, the cloned constructs were not active under different experimental conditions. Identifying the functionally active sugar response elements in the predicted promoter regions of genes that are targets of glucose signaling will improve our understanding of this regulatory process.

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CHAPTER ONE

INTRODUCTION

THE INFLUENCE OF SUGAR ON PLANT HOMEOSTASIS

In plants, sugars play an essential role as substrates for energy metabolism to sustain plant growth. Hence, sugar production, through photosynthesis, is a vital process. It has been well documented that plants are capable of adjusting their growth and development to maintain homeostasis (Koch *et al.* 1996; Jang *et al.* 1997; Moore *et al.* 2003; Rolland *et al.* 2006). For example, the balance between carbon assimilation and utilization is continually influenced by the plant's changing environmental conditions. Short periods of carbon starvation can result in growth inhibition, which is not immediately reversed upon carbon being made available (Usadel *et al.* 2008). Analyzing global transcriptional responses of *Arabidopsis thaliana* rosettes to a gradual depletion of carbon, Usadel *et al.* (2008) proposed that signaling events are initiated by small changes in carbon status. This type of sensitivity would require plants to be able to monitor sugar status and control gene expression to accommodate the constant changes in nutrient-dependent cellular activities. The plant's ability to sense the quality and quantity of varying sugars is important to develop an appropriate metabolic response to specific situations.

Cellular sugar status modulates and coordinates internal regulators and environmental cues that govern plant growth and development (Rolland *et al.* 2002). As

the products of photosynthesis are transported to developing plant organs, light and sugars regulate the particular growth activities of these specific structures. In most instances, low concentrations of sugars enhance photosynthesis and reserve nutrient mobilization and export, whereas an abundance of sugars promotes plant growth and carbohydrate storage (Rolland *et al.* 2002). Plants require coordinated modulation of gene expression and enzyme activities in both carbohydrate-exporting (source) and carbohydrate-importing (sink) tissues to ensure optimal synthesis and use of energy resources while adapting to the changing environmental conditions and nutrient availability. Plants can display photosynthesis, respiration, and fermentation at the same time in different tissues through a complex regulatory system that involves sugar signaling and integrates different metabolic, developmental, and environmental signals. (Rolland *et al.* 2002).

THE ROLE OF HEXOKINASE AS A SUGAR SENSOR IN PLANTS

Identifying the initial sugar sensing and response activating mechanisms in plants has been difficult due to a sugar's dual functions as a nutrient and a signaling molecule. Different experimental approaches, including screening mutants, developing signaling assays, and making targeted transgenics, have been used in attempts to identify the initial sugar sensing and signaling machinery.

Hexokinases have been identified as the primary glucose sensor in a broad range of organisms. Hexokinase (HXK) catalyzes the phosphorylation of hexose sugars at the first step of the glycolytic pathway. Most yeast and plant HXKs are approximately 50-54

kD in size. HXKs share a common conserved ATP binding site and a sugar binding domain, which are responsible for determining substrate affinities. It has been suggested that HXK sensing and signaling functions are dependent on HXK's subcellular localization (Balasubramanian *et al* 2007), possible translocation to the nucleus (Rolland *et al.* 2007), and/or interactions with downstream effectors (Cho *et al.* 2007).

In the Arabidopsis genome, there are three HXK genes (AtHXK1, AtHXK2, and AtHXK3) and three hexokinase-like genes (Karve *et al.* 2008). These six genes can be categorized as one of two types of HXKs based on their intracellular localization (Olsson *et al.* 2003). Type A kinases, including AtHXK3, have a predicted chloroplast transit peptide. Sequence analysis of these domains suggested that they represent an ancestral form of plant HXK predating the separation of mosses from higher plants. Type B kinases, including the other five Arabidopsis HXK and hexokinase-like proteins, contain an N-terminal hydrophobic membrane anchor. It is still not clear whether all six of the HXK and hexokinase-like genes have roles in glucose signaling.

Among plant HXKs, HXK1 in Arabidopsis is perhaps the best characterized glucose sensor. Studies using different sugars, sugar analogs, and metabolic intermediates in a mesophyll-protoplast transient expression system revealed that AtHXK1 is a core component in plant sugar sensing and signaling (Jang *et al.* 1994). To identify signaling components of intracellular pathways involved in glucose sensing and signaling, genetic strategies have been designed to independently select either glucose-insensitive or glucose-oversensitive mutants using detectable plant phenotypes.

Glucose insensitive (*gin*) mutants were recognized by overcoming the developmental arrest displayed by wild type seedlings that were grown on 6% glucose plus Murashige and Skoog (MS) medium (Figure 1.1). Sequencing identified that the *gin2-1* mutant contains a nonsense mutation (Q432stop*) while the *gin2-2* mutant contains a missense mutation (G416A), both within AtHXK1. Nonsense mutations can destabilize mRNA. Plants of *gin2-1* had reduced HXK1 but not HXK2 transcript levels (Moore *et al.* 2003). Furthermore, immunoblot analysis revealed that *gin2-1* is a HXK1 null mutant. The characterization of the *gin2-1* mutant provides evidence for a role of HXK1 as a glucose sensor/transducer which regulates gene expression and plant growth (Moore *et al.* 2003).

The plant's ability to sense and transduce glucose signals to regulate metabolism, growth, and stress responses are critical for survival. HXK1 has a unique function in a broad spectrum of glucose responses, including gene expression and plant growth. From an analysis of *gin2-1* phenotypes, Moore *et al.* (2003) suggested that the roles of HXK1 in growth promotion or growth inhibition depend on glucose concentration, cell type, developmental state, and environmental condition. The antagonistic and synergistic hormone-like effects of glucose on plant growth and development reflects the complex signaling network governed by nutritional and environmental inputs. The integration of these signals, in part, is through HXK1 regulatory functions.

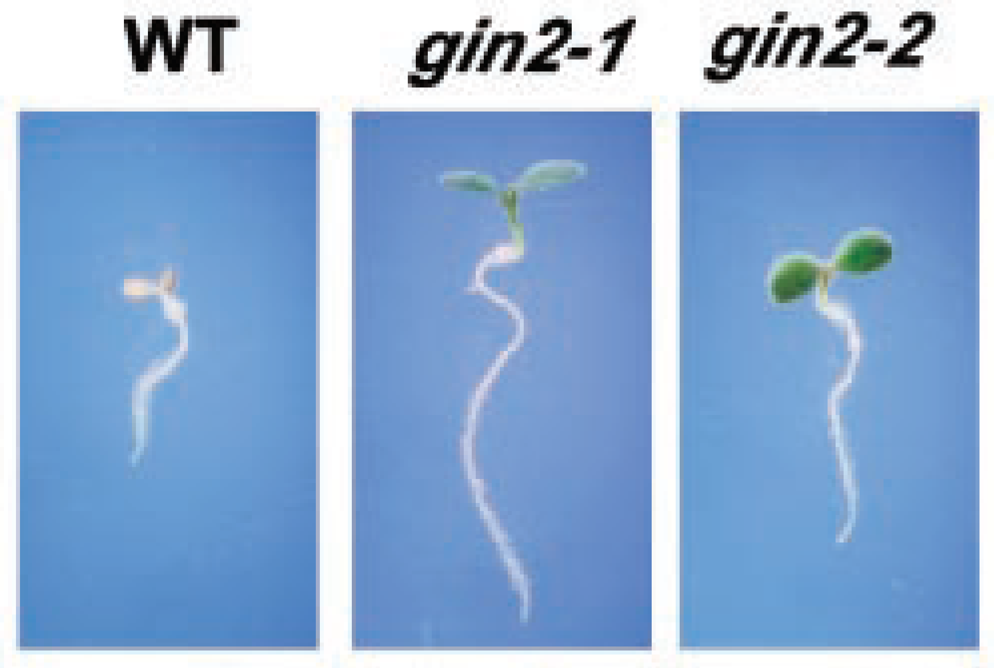

Figure 1.1: Glucose-insensitive *(gin)* mutants have been identified in *Arabidopsis thaliana.* When grown on agar plates containing 6% glucose + MS medium under light for 5 days, the wild type (WT) seedlings display arrested growth development. *Gin2-1* and *gin2-2* mutants were originally identified by their tolerance to glucose (Moore *et al.* 2003).

The role of HXK has helped identify three distinct glucose signal transduction pathways in plants (Xiao *et al.* 2000). In the first pathway, the HXK1-dependent pathway, gene expression is regulated by HXK1-mediated signaling function. A major effect of this pathway is the repression of photosynthetic gene expression. Interestingly, this can involve the actin cytoskeleton (Balasubramanian *et al.* 2007). In the second pathway, HXK enzyme activity promotes gene expression programs that respond to glucose metabolism. In the third pathway, gene expression is controlled by signaling pathways independent of HXK1 functions. One example of the latter is glucose induction of pathogenesis related proteins.

DESCRIPTION OF THE PLANT ACTIN CYTOSKELETON AND INTERACTIONS WITH G-PROTEINS

The actin cytoskeleton is a three dimensional structure contained within the cell's cytoplasm. This structure acts as both "muscle" and support structure by maintaining the cell's shape and enabling cellular expansion (Staiger 2000). For many cellular functions, the cytoskeleton is used as an active track by molecular motors of the myosin or kinesin/dynein families. However, reorganization of the actin cytoskeleton is also important for cellular transduction of effectors such as light, calcium, abscisic acid, and other hormones (Vantard and Blanchoin 2002).

The eukaryotic cytoskeleton can be divided into three major components: microfilaments, intermediate filaments, and microtubules. Microfilaments are composed of two intertwined chains of actin monomers (G-actin) polymerized into microfilaments

(F-actin). Microfilaments are concentrated just beneath the cell's membrane and are essential for many signal transduction processes (Staiger 2000). Microtubules are hollow tubes composed of alpha (α) and beta (β) subunits of the protein tubulin that act as cellular support beams, providing a set of "tracks" for cell organelles and vesicles to move on (Staiger 2000).

Many cellular processes are dependent on the spatial and temporal organization of actin, and are regulated by the assembly and disassembly of actin. GTP-binding proteins are considered important regulators of cytoskeletal assembly and organization and affect many aspects of eukaryotic cell function such as proliferation, and intracellular vesicle trafficking (Vernoud *et al.* 2003). GTPases function as molecular switches that cycle between 'active' and 'inactive' states, regulating the timing and specificity of events that take place within the cell (Figure 1.2). Structural and functional similarities between different members of this large superfamily have led to establishment of five distinct GTPase families: Ras, Rab, Rho, Arf, and Ran. Vernoud *et al.* (2003) identified 93 genes within four of these families in the Arabidopsis genome (Figure 1.3).

In Arabidopsis, all small GTPases that segregate with the Rho GTPases appear to be members of a unique subfamily only identified in plants. Because of this, they have been named Rop GTPases (for Rho-related proteins from plants). Given the conspicuous absence of the Ras family of GTPases, Vernoud *et al.* (2003) hypothesized that the ROP GTPases may reflect a unique mechanism that involves the actin cytoskeleton for control of cell signaling during plant development.

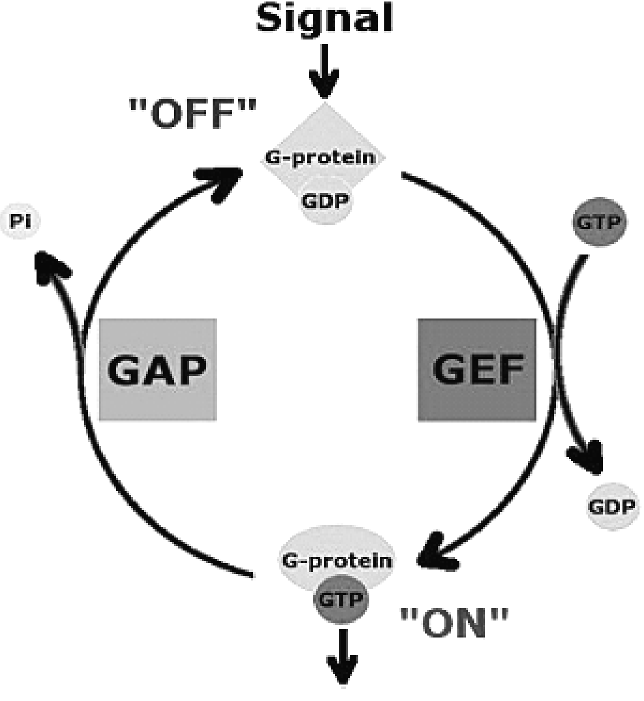

Figure 1.2: The GTPase activation cycle. GTPases are molecular switches that control many different cellular processes. By cycling between two different conformational states, the GTPases can regulate the timing and specificity of events that take place within the cell. Guanine nucleotide exchange factors (GEF) facilitate the binding of GTP to the GTPase. When bound to GTP, the GTPase is said to be in its ‘active’ state; capable of interacting with downstream effector proteins including various kinases and many scaffold-like or structural proteins. The GTPase remains active until the GTP is hydrolyzed to GDP, which is catalyzed by the GTPase Activating Protein (GAP). When bound to GDP, the GTPase is inactive (Vernoud *et al.* 2003).

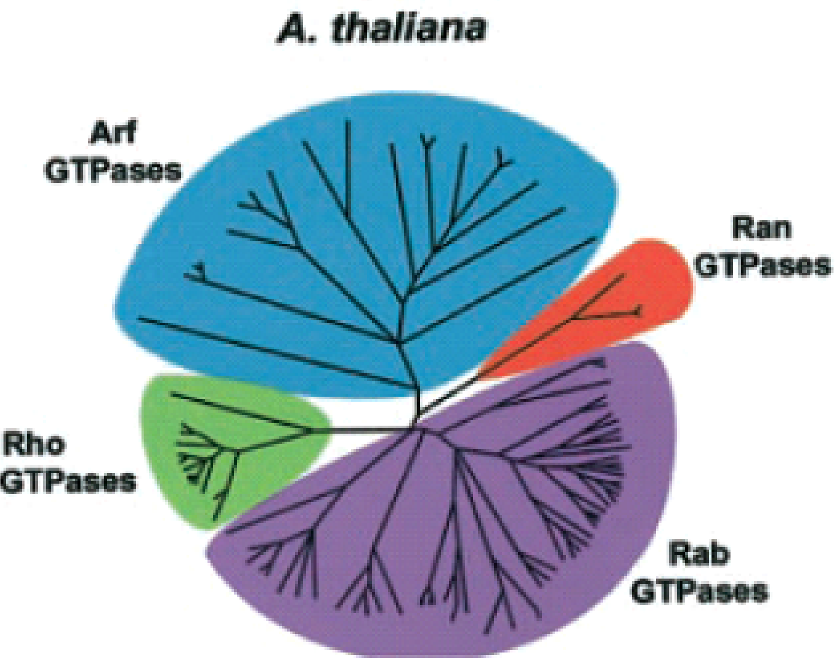

Figure 1.3: A phylogenetic star diagram of *A. thaliana* small GTPases (Vernoud *et al.* 2003). The Arabidopsis genome contains 57 Rab GTPases, 21 Arf GTPases, 11 Rho GTPases, 4 Ran GTPases, but no Ras GTPases.

The Presence of Glucose Disrupts the Functional Dynamics of the Plant Actin Cytoskeleton

Multiple stimuli, such as auxin, calcium, and abscisic acid, can affect the structure of F-actin. To determine long and short term affects of glucose treatment on F-actin organization in seedlings, Balasubramanian *et al.* (2007) used a transgenic line of *A. thaliana* expressing 35S:HXK1-GFP-*hTalin* as a reporter of F-actin dynamics. Seedlings grown on agar plates with 0.5% sucrose for 5 days displayed a large number of transverse and finer mesh actin filaments arranged in a grid-like pattern. When these seedlings were transferred to a solution of 1.8% (0.1 M) glucose, a rapid loss of many transverse and fine mesh filaments accompanied an increased bundling of the longitudinal F-actin cables (Figure 1.4 A). The re-organization of F-actin into fine mesh filaments after transferring the repressed seedlings from 6% glucose into a solution of water demonstrated that the disruptive effects of glucose on the F-actin filaments of the actin cytoskeleton could be reversed (Figure 1.4 B).

Balasubramanian *et al.* (2007) found that HXK1 can interact with F-actin both *in vitro* and *in vivo*. This was supported by coimmunoprecipitation assays from transgenic plants expressing HXK1-FLAG, which demonstrated a physical association between AtHXK1 and F-actin. To determine whether this interaction might have a functional significance, Balasubramanian *et al.* (2007) used *A. thaliana* leaf protoplasts to test known reagents that disrupt actin filaments (latrunculin-B and cytochalasin D) for their possible effects on HXK1-dependent glucose signaling. Transfection of HXK1 plus treatment with 2 mM glucose resulted in a 3-fold reduction in activities of 2 different

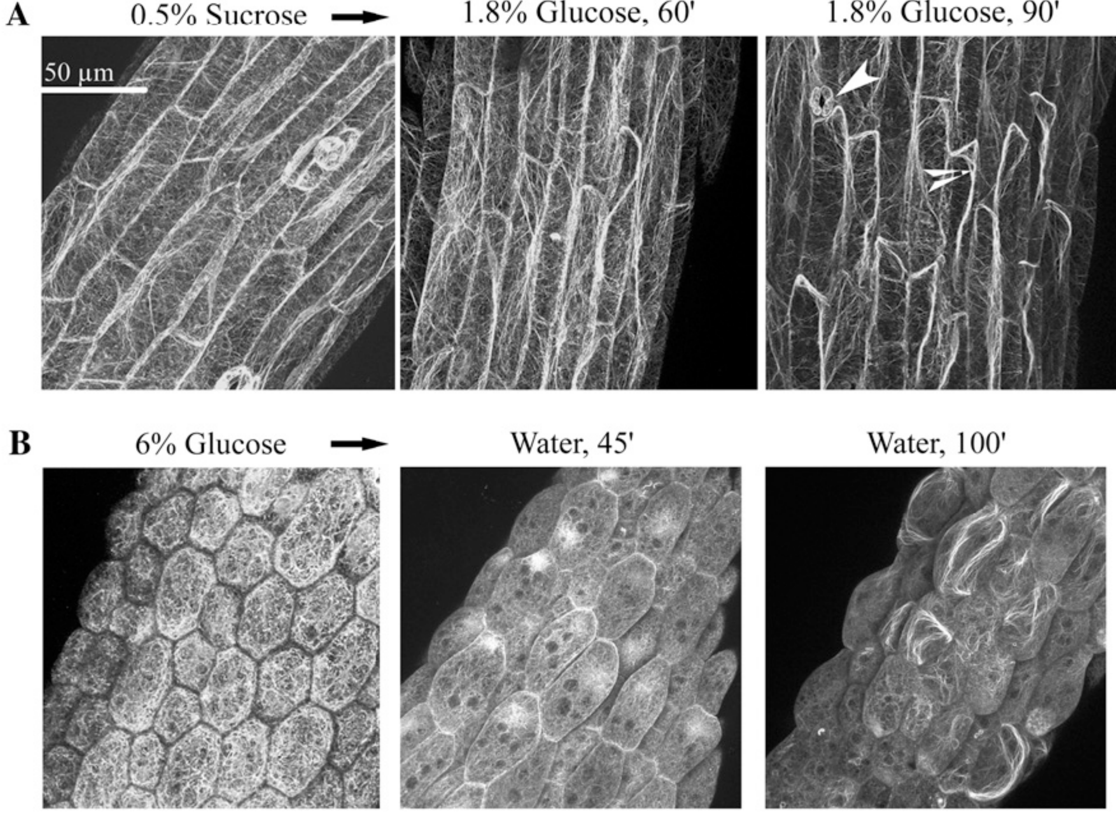

Figure 1.4: The effects of glucose on F-actin organization (Balasubramanian *et al.* 2007). **A.** After 5 days of growth on agar plates with 0.5% sucrose, *A. thaliana* seedlings were transferred to solutions of 1.8% glucose. Disruption of F-actin can be visualized in the hypocotyls of GFP-hTalin seedlings (arrow heads point to open stomate and an actin cable). **B.** Seedlings were grown on 6% glucose for seven days and then transferred to water for indicated times. Note the initial absence of fine mesh filaments before transfer and the beginning reorganization of F-actin after transfer to water

promoters in transient expression assays. The addition of latrunculin-B (LatB) or cytochalasin D (CytoD) did not affect promoter expressions in the absence of HXK1 and glucose, but did completely block effector-dependent repression of both promoter activities. Bioimaging revealed that LatB and CytoD treatment had extensively disrupted the finer actin filaments of leaf protoplasts.

MULTIPLE PLANT ACTIN BINDING PROTEINS ARE REPRESSED BY GLUCOSE

Actin is the major component of the cytoskeleton, existing largely as F-actin or with small amounts of G-actin as well. The rapid assembly and disassembly of the actin cytoskeleton is essential for many cellular functions. The dynamics of the actin cytoskeleton are tightly regulated *in vivo* by numerous actin binding proteins (ABPs) and the hydrolysis of ATP by actin (Pollard *et al.* 2003).

The vast majority of ABPs can be grouped into five distinct families using conserved actin-binding motifs (Dominguez 2004). These include, but are not limited to, the Wiskott-Aldrich syndrome recognized (WASP)-homology domain-2, the actin-depolymerizing factor/cofilin (ADF/cofilin) domain, the gelsolin-homology domain, the calponin-homology domain, and the myosin motor domain. Other domains will likely be recognized as more protein complexes involved in actin binding are identified. Interestingly, Price *et al.* (2004) found that of the nearly 1000 glucose regulated genes, gene transcripts of five actin associated proteins were repressed by glucose (Table 1.1).

The actin depolymerizing factor (ADF) is a small protein (~17 kDa) that is moderately conserved across eukaryotes. ADF functions in the remodeling of the actin

Table 1.1: Gene transcripts of several proteins associated with the re-organization of the actin cytoskeleton were transcriptionally repressed following a 3 h glucose treatment of Arabidopsis seedlings (Price *et al.* 2004).

Gene	Accession Number	Fold Change
Actin Depolymerizing Factor 9	At4g34970.1	4.1 ↓
Actin Related Protein 8	At5g56180.1	2.2 ↓
Rho GTPase Protein 1	At4g35750.1	6.1 ↓
Villin 3	At3g57410.1	2.6 ↓
Fimbrin 1	At4g26700.1	2.3 ↓

cytoskeleton by promoting the severing and depolymerization of actin at the growing ends of F-actin filaments (Bamburg *et al.* 1999). The number of ADF proteins varies among species, with plants normally having more ADF genes than animals. The *A. thaliana* genome contains 11 expressed ADF genes (Ruzicka *et al.* 2007). This gene family is characterized by the highly conserved single folded domain known as the ADF homology domain (Maciver and Hussey 2002).

Villin (VLN) is a 92.5 kDa tissue-specific actin-binding protein that shares structural homology with gelsolin (Friederich *et al.* 1999). VLN is composed of six evolutionarily conserved actin binding domains that constitute the core domain, followed by the carboxyl terminal headpiece domain. *In vitro*, villin caps, nucleates, severs, and bundles actin filaments in a calcium and phosphoinositide-dependent manner (Friederich *et al.* 1999). Both the headpiece and the core are capable of binding to F-actin, but to different sites on the actin filament. The overall structure of animal villin is highly conserved in Arabidopsis VLNs (AtVLNs). The major differences can be found in the portion of the protein that links the six actin-binding modules and the villin-specific headpiece domain. The specific and localized expression of mammalian VLN suggests that it has a distinct function in absorptive tissues. In plants, VLN is expressed ubiquitously in all tissues. However, the expression of certain VLN genes varies among different cell layers (Klahre *et al.* 2000).

cDNA probes homologous to mammalian VLN were used to screen a genomic library of *A. thaliana* and isolate full length clones. Sequence analysis revealed the Arabidopsis genome contains four genes encoding villin-like proteins. These four genes

can be subdivided into two groups, each containing two members. AtVLN2 and AtVLN3 share 84% nucleotide identity and encode proteins with 79% amino acid identity. AtVLN1 and AtVLN4 share a conserved headpiece domain, but the overall amino acid identity between the two proteins is only 56% (Klahre *et al.* 2000).

The actin-related protein (ARP) is not a recognized member of the ABP superfamily, but members of the ARP gene family have 60% amino acid sequence homology with conventional actins, sharing the conserved actin fold for the nucleotide binding domain. The nuclear ARPs function as essential components of chromatin remodeling and modifying complexes (Meagher *et al.* 2007). The ARP gene family is moderately conserved throughout eukaryotes and can be phylogenetically classified into six subgroups. Eight ARP gene sequences have been identified in *Arabidopsis thaliana* (*At*ARP 2-9; McKinney *et al.* 2002). Protein orthologs were identified in other species for the majority of the ARP gene sequences; however, ARP8 was suggested to be a novel protein that is plant specific. The transcripts from the ARP gene family members have distinct organ specific expression patterns, and the ARP proteins function in diverse cytoskeletal processes in both the cytoplasm and the nucleus, independently from actin. AtARP2 and AtARP3 transcripts are expressed at very low levels in all organs, AtARP5, AtARP6, and AtARP8 each have distinct transcript expression patterns in seedlings, roots, leaves, flowers, and siliques, while AtARP4 and AtARP7 proteins were shown to be most highly expressed in flowers (McKinney *et al.* 2002).

UNDERSTANDING THE TRANSCRIPTIONAL MACHINERY UNDERLYING SUGAR SIGNAL RESPONSES

Over the years, a large number of plant genes have been found to be transcriptionally regulated by sugars, consistent with the coordinated regulation of source and sink activities. However, little is known about the transcriptional mechanisms which underlying these responses. The functional dissection of sugar-induced gene promoters has led to most of the current progress made in understanding transcriptional responses. However, most of the information generated on regulatory *cis*-elements involved in sugar signaling comes from only a few genes.

The first sugar response element (SRE) was identified from sweet potato tuber (Ishiguro and Nakamura 1994). Located in the 5' upstream region of three different genes encoding for sporamin and α-amylase, the SP8 promoter element was found to be responsible for the sucrose induction of gene expression. Furthermore, the SPF1 transcription factor was shown to be a sucrose-repressed negative regulator that binds to the SP8a and SP8b promoter elements.

Sun *et al.* (2003) were interested in learning more about the SP8a element's involvement in sugar-mediated gene regulation. A probe was designed from an isolated cDNA clone of the barley SPF1 ortholog and used to screen an endosperm cDNA library. Restriction mapping and sequence analysis revealed that all the positive clones grouped into three distinct classes: SUSIBA1, SUSIBA2, and SUSIBA3. Amino acid alignment of the SUSIBA2 protein sequences identified two highly conserved WRKY domains that shared two zinc finger motifs involved in binding to the appropriate DNA elements.

Those authors suggested as one possibility that the C-terminal WRKY domain mediates sequence specific binding to DNA elements, whereas the N-terminal WRKY domain facilitates DNA binding and/or engages in protein-protein interaction.

Using electrophoretic mobility shift assays, Sun *et al.* (2003) demonstrated that SUSIBA2 binds to three sugar responsive elements (SURE-a, SURE-b, and SURE-c) and a W-box element. Based also on a later study, Sun *et al.* (2005) proposed a model for sugar signaling in which high sugar status in sink tissues maintains an active SUSIBA2 gene, permitting the SUSIBA2 transcription factor to bind and activate the SURE elements.

The binding of SUSIBA2 to the SURE elements displayed a novel feature for a WRKY protein. The WRKY proteins are a superfamily of transcription factors. The name of the WRKY family is derived from the most prominent feature of these proteins, the WRKY domain, a 60 amino acid region that is highly conserved among family members. The WRKY domain is defined by the conserved amino acid sequence WRKYGQK at its N-terminal end, together with a novel zinc-finger-like motif (Eulgem *et al.* 2000). Although there is a strong conservation of their DNA-binding domain, the overall structures of WRKY proteins are highly divergent.

Plant WRKY DNA binding proteins recognize various W-box elements with a TGAC core sequence. However, determining the roles of individual family members in regulating specific transcriptional programs during development or in response to environmental signals has been difficult because very little is known about the developmental programs that require the functions of WRKY proteins. One suggestion is

that WRKY transcription factors should regulate the temporal and spatial expression of specific genes, thereby ensuring the proper response to internal and external stimuli (Ulker and Somssich 2004).

Additional sugar regulated promoter sequences have been identified by extensive transcriptional studies of barley, wheat, and rice genes that encode for α-amylase (αAMY). Initial screening recognized two consensus sequences, TAACARA and TATCCAT, in the 5´ flanking regions of the αAMY genes, as *cis*-acting regulatory elements (Huang *et al.* 1990). These sequences eventually became recognized as the amylase box 1 (AMYBOX1) and amylase box 2 (AMYBOX2) response elements.

Lu *et al.* (1998) first identified a sugar response sequence in rice with three essential elements for sugar starvation-induced expression: the GC-box, the G-box, and the TATCCA element. The latter was eventually recognized as the "TATCCA"osamy element. Lu *et al.* (2002) later demonstrated that the TATCCA element is located approximately 100 nucleotides upstream of the transcription start site. They also demonstrated that three novel rice MYB proteins possess specific binding activity for the TATCCA sequence *in vitro*.

MYBs are a group of transcription factors with conserved DNA binding domains located on the N-terminal end of the gene product. Three different binding domains create three predominant MYB forms, designated R1, R2, and R3. Plants contain either the R2 or R3 form of the MYB transcription factor. *A. thaliana* contains 125 R2/R3 MYB genes, which are involved in numerous biological functions ranging from secondary metabolism, signal transduction, and disease resistance (Stracke *et al.* 2001).

Multiple databases have attempted to organize the recognized *cis*-acting regulatory DNA elements in a manner that is easily accessible to the general public. The Arabidopsis Gene Regulatory Gene Server (AGRIS), produced by the Ohio State University, is an information database pertaining to Arabidopsis promoter sequences, transcription factors and their targets. AGRIS is comprised of two separate databases, the *A. thaliana cis*-regulatory database (*AtcisDB*) and the *A. thaliana* transcription factor database (*AtTFDB*).

Another related database, The Plant *Cis*-acting Regulatory DNA Elements (PLACE), contains motifs identified in published reports. This includes 11 motifs identified as sugar response elements (Table 1.2). The associated PLACE Web Signal Scan program can be used to facilitate the analysis of DNA sequences which identify known response elements to various stimuli. As more is understood about how the signal transduction network operates on the molecular level, these databases will continue to expand and aid in assembling the missing puzzle pieces to this complex process.

Table 1.2: The PLACE database contains 11 previously identified sequence motifs that are classified as sugar response elements (SREs).

SREs	Sequence
"ACGT" A Box	ACGTA
"ACGT" C Box	ACGTC
"ACGT" T Box	ACGTT
Amylase Box 1	TAACARA
Amylase Box 2	TATCCAT
"CGACGT"osamy	CGACGT
Pyrimidine Box	CCTTTT
SRE	TTATCC
SUSIBA 2	TGACT
"TATCCA"osamy	TATCCA
WB Box	TTTGACY

IDENTIFYING FUNCTIONALLY ACTIVE SUGAR RESPONSE ELEMENT MOTIFS

Identifying the functionality of *cis*-acting regulatory sequences in gene promoters is challenging. Understanding how putative glucose regulatory elements modulate gene expression is not well understood and requires much more research. Identifying the functionally active sugar response elements in the predicted promoter regions of genes that undergo glucose induced transcriptional repression will lead us closer to understanding the signal transduction process at the molecular level. RT-PCR will be used to identify candidate genes for our study. The AGRIS database will be used to identify the promoter regions of these genes, while the PLACE database will help identify the *cis*-acting response elements found in these sequences. Unique response element motifs will be examined for functional activity using the protoplasts transient expression assay system. The applications of the protoplast transient expression system have contributed to our understanding of the functions of *cis*-acting regulatory elements and *trans*-factors in many different signaling pathways. Coupled with genetic, genomic, and transgenic approaches, these protoplast studies have helped unravel the control mechanisms of the essential *cis*-acting regulatory elements and transcription factors for light, phosphate, sugar, and cell cycle regulation in maize, parsley, and tobacco protoplasts (Sheen 2001).

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CHAPTER TWO

IDENTIFYING POSSIBLE SUGAR RESPONSE ELEMENTS IN THE PREDICTED PROMOTER REGIONS OF KEY ACTIN-REMODELING GENES FROM ARABIDOPSIS

ABSTRACT

To gain a better understanding of the glucose signaling network in plants, we first examined expression of five genes associated with the remodeling of the actin cytoskeleton that had been previously implicated as targets of glucose repression. Monitoring gene expression by RT-PCR indicated that only two of the five genes, ADF9 and ARP8, are glucose repressed. Screening for glucose induced transcriptional repression in other members of the ADF and ARP gene families revealed this form of repression was unique to the ADF9 and ARP8 genes. We then evaluated predicted sugar response elements in the promoters of these two genes. A four repeat amylase box 1 motif might be responsible for the observed glucose dependent repression of ARP8. However, a unique response element motif was not readily identified in the predicted promoter region of ADF9. Perhaps glucose induced repression of ADF9 occurs through a combination of known or unknown sugar response elements.

INTRODUCTION

The specific mechanisms of action behind sugar signaling responses on the molecular level remain unclear. It has been suggested that *cis*-acting response elements may be used by signaling molecules involved in regulating transcriptional responses (Higo *et al.* 1999). Recent gene promoter studies have identified specific response elements associated with different forms of external stimuli, including but not limited to sugar, ethylene, and ABA (Davuluri *et al.* 2003). Characterizing the promoter regions of genes that undergo transcriptional regulation has provided insight to the means by which external stimuli influence gene transcription.

During plant growth and development, the availability of sugars can modulate a wide range of vital plant processes. The initial idea of a linear transduction pathway in plants for regulating complex processes, such as sugar signaling, has begun to change. It has been proposed that multiple signal transduction pathways exist in plants (Xiao *et al.* 2000). Genetic screens using detectable plant phenotypes and transgenic seedlings carrying reporter and selection genes under the control of sugar regulated promoters have identified mutants with overlapping phenotypes. Characterization of these mutants revealed extensive connections between the signaling pathways for sugars and for two plant stress hormones, ethylene and abscisic acid (ABA); (Leon and Sheen 2003). Glucose signaling is generally antagonistic to ethylene signaling, yet requires elements of ABA signaling.

To gain a better understanding of the complex sugar signaling networks in plants, and the role of glucose as both signaling molecule and metabolite, Price *et al.* (2004) developed a microarray screen to identify the influence of glucose on global gene expression. Using a thresh hold value of 2-fold, their data indicated that expression of nearly 1000 genes was influenced by glucose, including repression of several ethylene biosynthetic genes.

The microarray data of Price *et al.* (2004) also indicated that the expression of five genes associated with the remodeling of the actin cytoskeleton (ADF9, ARP8, RGAP1, VLN3, and FIM1) was repressed. This data extends the observations of Balasubramanian *et al.* (2007), who showed that glucose rapidly and reversibly disrupts the formation of fine actin filaments (Figure 1.4).

Plotting the response elements recognized in the predicted promoters of genes that undergo glucose induced repression may identify unique motifs that might be involved in signaling this specific form of repression. Identifying these *cis*-acting response elements could provide insight to the molecular mechanisms behind sugar signaling and their influence on gene transcription.

MATERIALS & METHODS

RT-PCR

Arabidopsis seedlings were grown in liquid culture for 10 days under constant light with shaking, dark adapted for 24 hours, then treated with 2% glucose for 4 hours. These treatment conditions were similar, but not identical to those used by Price *et al.* (2004). cDNA, a gift of Penny Xia, was produced from RNA extracted from treated or control seedlings. Once template concentrations were established for balanced expression of a control gene (ubiquitin 5, UBQ5), respective cDNA amounts were kept constant (2.0 – 2.2 µl of depending on the sample) during the PCR reactions using other primer sets, while varying the number of PCR cycles as needed. During all RT-PCR reactions, a melting temperature of 94.0˚ C was held for 30 seconds, an annealing temperature of 55.0˚ C was held for 30 seconds, and an extension temperature of 68.0˚ C was held for 50 seconds. cDNA transcripts were used as a template for the 10 µl RT-PCR reaction containing 10 ng of the appropriate forward and reverse primers (Table 2.1) and 5 µl of 2x Taq Master Mix (New England Biolabs). PCR product bands were resolved on a 1.5% agarose gel (US Biological) containing ethidium bromide. Gel images were captured using a UV light source and the PCR products were quantified using Image J (http://rsb.info.nih.gov/ij/). Image J values were scored by multiplying the area of measurement by the mean pixel density, then subtracting the background measured from an equivalent blank gel area.

Table 2.1: The gene specific primers for RT-PCR. An asterisk indicates genes reported to be glucose repressed (Price *et al.* 2004).

cDNA Transcript (Locus)	Primer	Primer Sequence	Product Size
ACT2	ACT2RTF	5'-CTCCGCTTTGAATTGTCTCGTTGTC-3'	317 bp
(At3g18780)	ACT2RTR	5'-TGATGTCTTGGCCTACCAACAACAC-3'	
ACT7	ACT7RTF	5'-AATGGCCGATGGTGAGGATATTCAG-3'	525 bp
(At5g09810)	ACT7RTR	5'-TGAGGAAGAGCATACCCCTCGTAGA-3'	
ADF1	ADF1RTF	5'-GTTGGTCAACCGATCCAAACTTACG-3'	457 bp
(At3g46010)	ADF1RTR	5'-CAACATCGAACACAAGACCGAAACA-3'	
ADF2	ADF2RTF	5'-TCGGATAGCTTGCAAATAACGCTGA-3'	257 bp
(At3g46000)	ADF2RTR	5'-GGTACGGAATGTTCTTTTCGCCTTC-3'	
ADF5	ADF5RTF	5'-CGTGTGACGGATGAGTGTACGAGTT-3'	303 bp
(At2g16700)	ADF5RTR	5'-CCCATCTTTCGACGTTGCGTACAAT-3'	
ADF6	ADF6RTF	5'-CGACGCATCCGATTTTATTTGTTTG-3'	373 bp
(At2g31200)	ADF6RTR	5'-TTCGCTCGTTCGCGTAACACTTC-3'	
ADF9*	ADF9XF	5'-CTTTGCCGGAGGATGACTGTCGTT-3'	197 bp
(At4g34970)	ADF9XR	5'-GGTGGCTTGAAGCTGGTAGTGAACA-3'	
ARP2	ARP2RTF	5'-ACGGCACCGGTTATGTAAAATGTGG-3'	307 bp
(At3g27000)	ARP2RTR	5'-GGATTAAGAGGTGGATCCGTGAGCA-3'	
ARP3	ARP3RTF	5'-TGGGGCAACTCATGTTGTACCTG-3'	576 bp
(At1g13180)	ARP3RTR	5'-TCCACCGGTTGAGACGTAATTTCAC-3'	
ARP5	ARP5RTF	5'-GCAACCCAGTTCAATCTCGTAGCA-3'	519 bp
(At3g12380)	ARP5RTR	5'-CTCGCAGCCTTTGACCTTGTTTTTC-3'	
ARP8*	ARP8XF	5'-TGGAACCGATCGAATAGTGGCAAG-3'	668 bp
(At5g56180)	ARP8XR	5'-GCAGGGACGTTCATGTCAAACAAG-3'	
VLN3*	VLN3XF	5'-TGCAAGGGAATTCCTACCAGAAGAA-3'	604 bp
(At3g57410)	VLN3XR	5'-CTGCGTTTGAAGTCAATCCCTGTC-3'	
RGAP1*	RGAPXF	5'-CACCTGCGGCCGATTTCTATTTTC-3'	170 bp
(At4g35750)	RGAPXR	5'-TCGCTTTCTTGACCGTAATCCATCA-3'	
FIM*	FIM1XF	5'-CTGTGACCCGGCTACACTAGATGC-3'	629 bp
(At4g26700)	FIM1XR	5'-TGTTGGCCCAGCTGAGGATATCTG-3'	
UBQ5	UBI5F	5'-GTGGTGCTAAGAAGAGGAAGA-3'	250 bp
(At3g62250)	UBI5R	5'-TCAAGCTTCAACTCCTTCTTT-3'	

DEFINING THE GENE PROMOTER SEQUENCES AND IDENTIFYING RELEVANT RESPONSE ELEMENTS

The Arabidopsis Gene Regulatory Gene Server (AGRIS, http://arabidopsis.med.ohio-state.edu) is a database for Arabidopsis promoter sequences, transcription factors and their target genes. AGRIS is comprised of two separate databases, the *A. thaliana cis*-regulatory database (*AtcisDB*) and the *A. thaliana* transcription factor database (*AtTFDB*). The *AtcisDB* was searched using the TAIR Locus ID for the genes of interest. The results were examined to ensure the promoter sequences extended from the 3' end of the upstream gene and included the 5' UTR of the gene of interest.

The Plant Cis-acting Regulatory DNA Elements (PLACE, http://www.dna.affrc.go.jp/PLACE/signalscan.html) is a database of motifs found in plant *cis*-acting regulatory DNA elements, compiled from those identified in published literature through February 2007. By submitting the AGRIS predicted promoter region sequence into the Signal Scan Search component of the PLACE database, the cis-acting response elements located on the plus (+) and minus (-) stands of the predicted promoter regions of the *A. thaliana* ADF and ARP gene family members were identified.

RESULTS

GLUCOSE TREATMENT REPRESSES ADF9 AND ARP8 TRANSCRIPT ABUNDANCE

RT-PCR was used to investigate whether glucose represses transcription of the five actin associated genes previously identified by Price *et al.* (2004) to be down-regulated by glucose (Figure 2.1). UBQ5, a house keeping gene expressed in all plant tissues, was used to normalize the RT-PCR product using different template quantities.

Using Image J to quantify the RT-PCR results, the data suggested that glucose repressed gene transcription of the ADF9 and ARP8 samples at 28 and 30 cycles (Table 2.2). Although the Image J scores represent abstract values, they are products of pixel density measurements. In this study, they represent gene transcript abundances. The data suggests a transcript abundance difference of almost two fold can be seen in the reported values for the ADF9 and ARP8 RT-PCR gene transcript measurements at 28 and 30 cycles. In their microarray study, Price *et al.* (2004) reported transcription repression fold values of 4.1 and 2.2 for ADF9 and ARP8 respectively. Price *et al.* (2004) also reported glucose repression for the FIM, RGAP1, and VLN3 transcripts. However, we did not observe transcript repression for these genes in our RT-PCR study. This difference may have arisen from our study using seedlings grown in liquid culture medium, while the Price *et al.* (2004) study used plate grown seedlings that were placed into liquid solution for the glucose treatments.

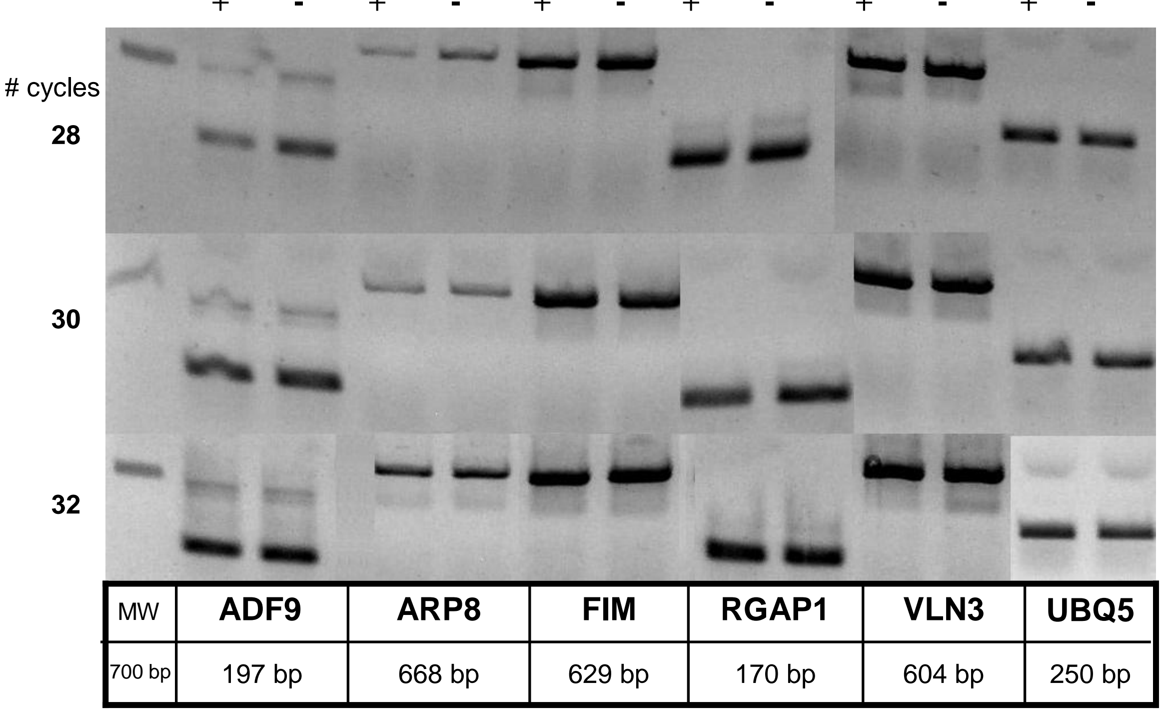

Figure 2.1: RT-PCR products after 28, 30, and 32 cycles were visualized for possible glucose induced transcriptional repression. For each gene transcript of interest, lane 1 contains samples exposed to glucose (+glc) while lane 2 contains samples free of glucose (-glc). The difference in transcript abundance seen in the ADF9 and ARP8 samples suggests that glucose induced transcriptional repression of these genes. The lower box indicates the expected PCR product size.

Table 2.2: The analytical results produced by Image J support the RT-PCR visual findings. Transcript abundance after 28, 30, and 32 cycles were measured using the capabilities of Image J. A difference of almost 2 fold was seen in the ADF9 and ARP8 transcripts.

# Cycles	Treatment	ADF9	ARP8	FIM	RGAP1	VIL3	UBQ
28	+glc	**31**	**14**	63	78	67	58
	-glc	**50**	**27**	72	85	78	52
30	+glc	**33**	**15**	68	84	71	62
	-glc	**53**	**29**	78	91	83	55
32	+glc	90	57	102	107	102	80
	-glc	91	65	106	104	101	75

Glucose Induced Repression is not Common Amongst ADF and ARP Gene Family Members

To examine if glucose also influenced the transcriptional expression of other members of the ADF and ARP gene families, a similar RT-PCR based approach was employed (Figure 2.2). Image J was used to quantify the amplified PCR products and verify the visual findings (Table 2.3 and Table 2.4). There was no significant difference in the Image J values reported for the ADF 1, 2, 5, and 6 mRNA, or for ARP 2, 3, and 5 family members after 28 and 32 cycles. Two actin gene samples, ACT 2 and 7, were also screened for possible glucose induced repression (Figure 2.2). Expression of these genes was also not influenced by the presence of glucose (Table 2.5). Therefore, glucose induced gene repression appears to be a unique feature of the ADF9 and ARP8 genes.

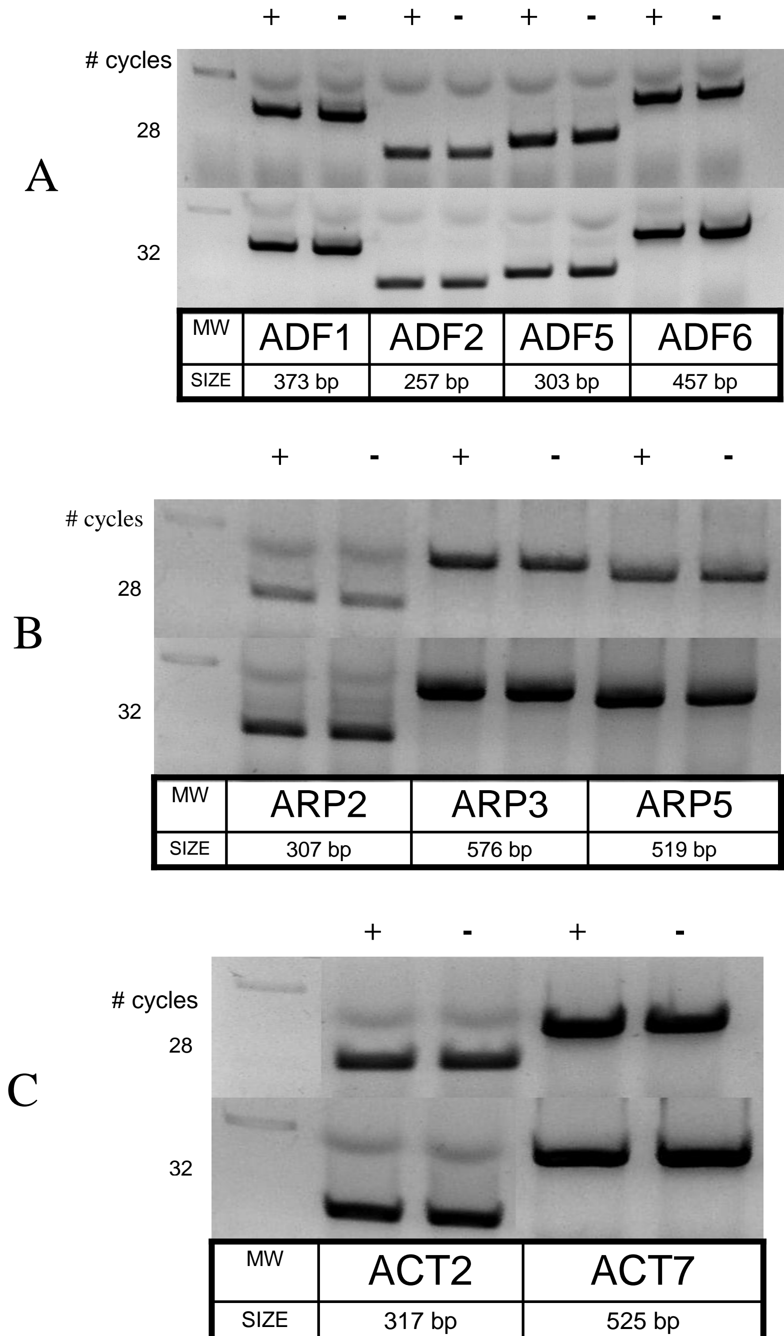

Figure 2.2: RT-PCR was used to screen multiple gene families for glucose induced transcriptional repression. Results for four of the eleven AtADF genes (ADF1, ADF2, ADF5, ADF6 of the AtADF) (**A**), three of the eight AtARP genes (ARP2, ARP3, ARP5) (**B**), and two actin genes (ACT2, ACT7) (**C**) were examined. For each transcript of interest, lane 1 contains samples exposed to glucose (+glc) while lane 2 contains samples without glucose (-glc). The data indicates that none of these genes undergo glucose induced repression.

Table 2.3: Screening members of the ADF gene family for glucose induced repression with Image J. Quantifying the RT-PCR data with Image J verified that glucose does not repress expression of any of those ADF family members screened.

# Cycles	Treatment	ADF1	ADF2	ADF5	ADF6
28	+glc	202	155	227	213
	-glc	202	132	209	193
32	+glc	249	207	229	254
	-glc	272	195	238	276

Table 2.4: Screening members of the ARP gene family for glucose induced repression with Image J. Quantifying the RT-PCR data with Image J verified that glucose does not repress expression of any of those ARP family members screened.

# Cycles	Treatment	ARP2	ARP3	ARP5
28	+glc	42	89	72
	-glc	41	81	72
32	+glc	136	173	159
	-glc	139	171	154

Table 2.5: Screening members of the actin gene family for glucose induced repression with Image J. Quantifying the RT-PCR data with Image J verified that glucose does not repress expression of either of those actin family members screened.

# Cycles	Treatment	ACT2	ACT7
28	+glc	194	263
	-glc	201	254
32	+glc	191	249
	-glc	185	260

IDENTIFYING RESPONSE ELEMENTS IN THE PROMOTER REGIONS OF INTEREST

The predicted promoter regions of the five actin associated genes reported by Price *et al.* (2004) to be glucose repressed were identifed using the AGRIS *Atcis*DB. The sequences were then entered into the Signal Scan Search component of PLACE, and the sugar response elements (SREs) were identified (Table 2.6). The data collected for each response element consisted of its orientation on either the (+) or (-) strand of DNA as well as its absolute value of base pairs upstream of the gene's ATG translation start site. The "ACGT" boxes do not contain a (+) or (-) strand orientation as a result of their palindromic sequence.

SREs were identified in each of the five predicted promoter sequences with 5 to 37 total SREs identified in a given sequence (Figure 2.6). The two genes indicated by RT-PCR to be repressed by glucose, ADF9 and ARP8, contain the least number of total SREs, 5 and 11 respectively. The predicted promoter sequence for the ARP8 gene was the only sequence to contain 4 repeats of the amylase box 1 motif, which spans approximately 300 bp on the (+) strand.

The predicted promoter regions for the entire *A. thaliana* ADF and ARP gene family members were also identified (See Appendix A) and characterized in a similar fashion (Table 2.7 and Table 2.8 respectively). The predicted promoter regions for all of the ADF and ARP gene family members contain at least one SRE sequence, while the majority contains a combination of different SREs. Once again, the 4 repeats of the amylase box 1 motif was recognized exclusively in the predicted promoter sequence of ARP8 and not in the other family members.

Recent studies have supported the concept of signal cross talk between sugar and ethylene signaling (Leon and Sheen 2003). The PLACE database recognizes seven possible ethylene response elements (EREs). Of the seven recognized EREs, only three ethylene response elements were identified in those predicted promoter sequences (Table 2.9). Initially identified in tomato was identified in the majority of the predicted promoter sequences, including ADF9 and ARP8. The core *cis*-binding element in tomato cysteine protease (LeCp) was also identified in the majority of the predicted promoter sequences, including ARP8. The "AGC box", an enhancer element identified in tobacco, was only recognized in the predicted promoter sequence of ADF11. However, there were no distinguishable EREs unique to the ADF9 or ARP8 predicted promoter sequences.

Table 2.6: The sugar response elements (SREs) recognized by PLACE Signal Scan Search in the predicted promoter regions of 5 actin-associated genes reported by Price *et al.* (2004) to be glucose repressed. The total number of response elements identified in the predicted promoter regions, as well as where, on either the + or – strand, each response element is located is recorded.

SREs	ADF9 695 nt	ARP8 1016 nt	FIM 2990 nt	RGAP1 3002 nt	VLN3 1505 nt
"ACGT" BOX	1x*		2x*		1x*
	657		1964, 2486		705
AMYLASE BOX 1		4x		1x	1x
		355+, 401+, 531+, 656+		1997-	345-
AMYLASE BOX 2		1x	1x	3x	
		491+	649+	1053-, 1120-, 2296+	
"CGACGT" Osamy	1x	1x	1x	5x	2x
	15+	78+	2243+	2311-, 2510-, 2694-, 2386+, 2389+	395-, 1027-
PYRIMIDINE BOX	1x	2x	6x	5x	5x
	366-	385-, 942-	885+, 957-, 1238-, 1513+, 1691+, 1923+	28-, 85-, 165-, 1940+, 2040+	243-, 312+, 447+, 506-, 674-
SRE				7x	2x
				235+, 402+, 1051-, 2297+, 2681+, 2927-, 2948-	316+, 1172+
SUSIBA2	2x	1x	7x	9x	3x
	431+, 526-	102-	26-, 385+, 1796+, 2437+, 2595-, 2625-, 2670+	171-, 575-, 921+, 1507-, 1594+, 1725+, 2396+, 2606-, 2937+	174+, 971+, 1500-
"TATCCA" osamy		2x	1x	5x	1x
		490-, 879+	649+	234+, 337-, 1052-, 1119-, 2296+	1171+
WB BOX			1x	2x	2x
			2439+	171-, 2398+	973+, 1500-
TOTAL	**5**	**11**	**19**	**37**	**17**

Table 2.7: The sugar response elements (SREs) recognized by PLACE Signal Scan Search in the predicted promoter regions of the ADF gene family.

SREs	ADF 1 1691 nt	ADF 2 684 nt	ADF 3 3002 nt	ADF 4 800 nt	ADF 5 2908 nt	ADF 6 373 nt	ADF 7 495 nt	ADF 8 947 nt	ADF 9 695 nt	ADF 10 3002 nt	ADF 11 1128 nt
"ACGT" BOX			1x*		1x*	2x*			1x*	1x*	1x*
			2579		2303	246, 339			657	1021	425
AMYLASE BOX 1	2x	2x	1x		2x	1x				4x	
	858-, 1131+	109+, 629-	2972+		103+, 2204-	200-				272-, 563+, 1845+, 2854-	
AMYLASE BOX 2		1x			1x					1x	
		73+			2003-					633-	
"CGACGT" Osamy	3x				4x	2x	2x	1x	1x		
	258+, 923-, 1180+				134+, 375+, 1672-, 2301-	244-, 247+	8-, 242-	105+	15+		
PYRIMIDINE BOX	2x	1x	3x	1x	5x	1x	1x	1x	1x	7x	
	580+, 866+	458+	638+, 1132-, 1440+	653+	1366-, 2237+, 2568+, 2720-, 2800-	229+	23-	370-	366-	116-, 347+, 400-, 792+, 2113+, 2165-, 2680+	
SRE		2x			2x						2x
		74+, 191+			1728-, 2001-						341-, 508-
SUSIBA2	2x		5x		2x	1x	1x	2x	2x	7x	
	1437-, 1579+		1462-, 1534-, 2012+, 2928-, 3001+		861+, 1009+	351-	289-	88-, 279-	431+, 526-	1148+, 1412+, 2373+, 2455-, 2539-, 2842-, 2951+	
"TATCCA" osamy		2x	1x		3x					1x	1x
		73+, 190+	1046-		1480+, 1729-, 2002-					632-	342-
WB BOX	1x	1x	3x					1x		1x	1x
	577+	326+	518-, 1066-, 2928-					279-		2953+	180-
TOTAL	**10**	**9**	**14**	**1**	**20**	**7**	**4**	**5**	**5**	**22**	**5**

Table 2.8: The sugar response elements (SREs) recognized by PLACE Signal Scan Search in the predicted promoter regions of the ARP gene family.

SREs	ARP2 2942 nt	ARP3 2637 nt	ARP4 198 nt	ARP5 360 nt	ARP6 732 nt	ARP7 146 nt	ARP8 1016 nt	ARP9 810 nt
"ACGT" T BOX	1x*	1x*						
	1246	1086						
AMYLASE BOX 1	1x	1x				1x	4x	1x
	2584+	568-				140-	355+, 401+, 531+, 656+	17+
AMYLASE BOX 2					1x		1x	
					226-		491+	
CGACGTosamy	3x		1x				1x	
	62+, 2547+, 2704+		76+				78+	
PYRIMIDINE BOX	6x	3x	1x	1x			2x	
	138-, 887+, 1656+, 1767-, 1871-, 2924+	251+, 2161-, 2408-	153-	313-			385-, 942-	
SRE		1x						
		1829+						
SUSIBA2	11x	4x		1x			1x	
	479+, 538-, 742+, 801+, 976-, 1038+, 1069-, 2317+, 2322-, 2501+, 2622-	813+, 1022+, 1670-, 2376-		52-			102-	
TATCCAosamy	1x	1x			1x		2x	1x
	2464+	185+			225-		490-, 879+	339+
WB BOX	4x							
	210+, 976-, 1040+, 1069-							
TOTAL	**27**	**11**	**2**	**2**	**2**	**1**	**11**	**2**

Table 2.9: The ethylene response elements (EREs) recognized by PLACE Signal Scan Search in the predicted promoter regions of the ADF and ARP gene families.

EREs	ERE	AGC Box	LeCp	TOTAL
ADF1	4		1	5
ADF2				0
ADF3	1		2	3
ADF4				0
ADF5	3		1	4
ADF6	1			1
ADF7				0
ADF8			1	1
ADF9	1		1	2
ADF10	1			1
ADF11		2		2
ARP2	1			1
ARP3	2		1	3
ARP4				0
ARP5				0
ARP6				0
ARP7	1			1
ARP8	1			1
ARP9	1			1
FIM	1		2	3
RGAP1			1	1
VLN3			2	2

Characterizing the Predicted Gene Promoter Regions of Interest

Different combinations of the eleven recognized SREs were identified among the predicted promoter regions of interest using the Signal Scan Search component of the PLACE database. Various symbols were designed to illustrate the results (Table 2.10). Using these symbols, the predicted promoter regions of the ADF (Figure 2.3) and ARP (Figure 2.4) family members were mapped.

The predicted promoter region of the ADF9 gene contains five SREs: one "AGCT" T-Box motif, two SUSIBA 2 motifs, one pyrimidine box, and one "CGACGT"osamy motif. Similar locations and orientations of these response elements could also be found in the predicted promoter regions of other ADF genes that did not undergo glucose induced transcription repression, making it difficult to identify distinguishable features of the ADF9 predicted promoter region.

The predicted promoter region of the ARP8 gene contains eleven SREs: two pyrimidine boxes, two "TATCCA"osamy motifs, four amylase 1 boxes, one amylase 2 box, one susiba 2 motif, and one "CGACGT"osamy motif. Although a commonality can be seen among some of the SREs identified by the PLACE database in the other ARP gene family members, there was a distinguishable feature unique to the predicted promoter sequence of ARP8. Four repeats of the amylase box 1 motif were located approximately 500 nucleotides upstream of the ATG translation start site (roughly half-way through the predicted promoter region), spanning approximately 300 nucleotides on the (+) strand of the predicted promoter region. This motif repeat was not identified in any of the other predicted promoter regions of the ADF or ARP family members (Figure

Table 2.10: The 11 sugar response elements (SREs) recognized by PLACE, with corresponding mapping symbols.

SRE	Sequence	Symbol
"ACGT" A Box	ACGTA	
"ACGT" C Box	ACGTC	
"ACGT" T Box	ACGTT	
Amylase Box 1	TAACARA	
Amylase Box 2	TATCCAT	
"CGACGT"osamy	CGACGT	
Pyrimidine Box	CCTTTT	
SRE	TTATCC	
Susiba 2	TGACT	
"TATCCA"osamy	TATCCA	
WB Box	TTTGACY	

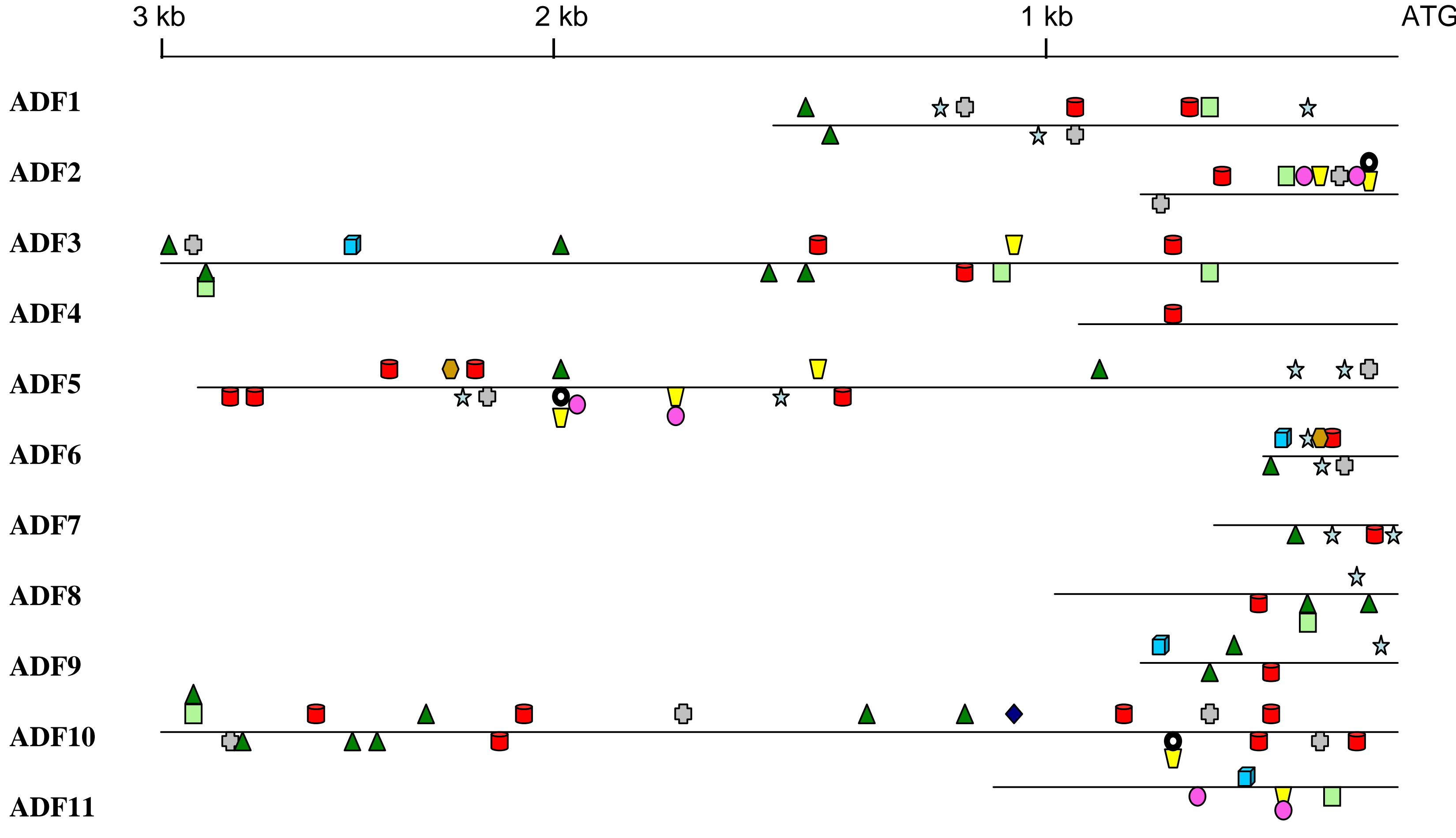

Figure 2.3: The sugar response elements identified in the AGRIS predicted gene promoters of the *Arabidopsis thaliana* ADF gene family by the PLACE Signal Scan Search program. See Table 2.10 for explanation of symbols.

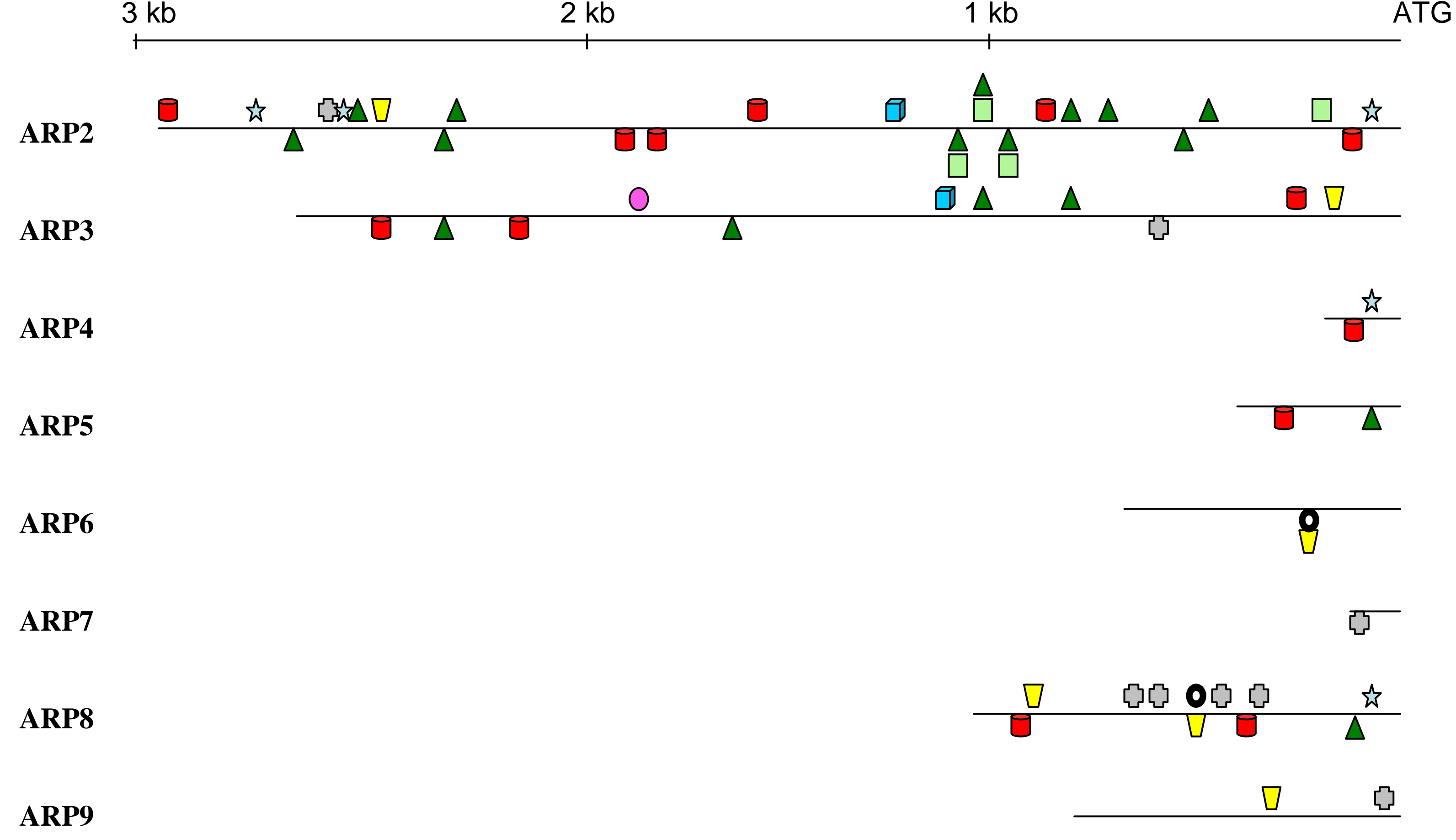

Figure 2.4: The sugar response elements identified in the AGRIS predicted gene promoters of the *Arabidopsis thaliana* ARP gene family by the PLACE Signal Scan Search program. See Table 2.10 for explanation of symbols.

2.3 and Figure 2.4, respectively), nor was it recognized in any of the other genes associated with actin remodeling that the Price *et al.* (2004) microarray study found were repressed by glucose (Table 2.6).

DISCUSSION

THE PREDICTED PROMOTER REGIONS OF THE ADF9 AND ARP8 GENES CONTAIN SUGAR SIGNALING RESPONSE ELEMENTS

Microarray data showed that glucose rapidly induced repression of five genes associated with actin cytoskeleton remodeling (Price *et al.* 2004). We confirmed by RT-PCR that glucose does repress transcription of the ADF9 and ARP8 genes, but our data did not show repression of FIM, RGAP1, and VLN3 (Figure 2.1). This difference may have been the result of differences in seedling growth conditions between the studies. Therefore we focused on the predicted promoter sequences of the ADF9 and ARP8 genes to gain a better understanding of which *cis*-acting elements might mediate glucose signaling on the molecular level.

The predicted promoter region of the ADF9 gene contains five previously identified SREs (Figure 3.3), while the predicted promoter region of the ARP8 gene contains 11 SREs (Figure 3.4). The predicted promoter regions of the ADF and ARP gene families were also characterized in a similar fashion to identify if the presence or proximity of any of the sugar response elements identified in the ADF9 or ARP8 predicted promoter regions were unique to these genes.

The ARP8 predicted promoter region contained a single distinguishable response element repeat motif, four repeats of the amylase 1 box motif. This repeat motif was not found in any of the other samples tested for glucose induced repression, nor was it identified in any of the SRE characterizations of the predicted promoter regions of the ADF or ARP gene family members. Thus, this repeat might be important for glucose signaling.

Although the ADF9 predicted promoter region contained multiple SREs, specific features unique to this region were not apparent. Many of the SREs identified in the predicted promoter region of the ADF9 gene were also found in similar locations and orientations in other predicted promoter regions of genes that did not undergo glucose induced repression. These results suggest that glucose induced repression of ADF9 might result by signaling through a combination of SREs and/or other hormone signaling response elements.

The proximity of a response element to the ATG translation start site might also be an important factor. Functionally active response elements have commonly been identified in the first kilobase of sequence upstream of the ATG translation start site. Focusing this study to the aforementioned region, rather than examining the entire AGRIS predicted promoter sequence, is another strategy to identify unique features in the predicted promoter sequence of the ADF9 gene that might facilitate glucose signaling on the molecular level. However, this analysis also did not identify unique motifs in ADF9. Perhaps other important motifs have not been identified.

INTERPRETING THE PLACE RESULTS

Scoring the predicted promoter regions for the individual SREs required some interpretation. The data produced by the Web Signal Scan of the PLACE database identified previously published response elements in the (+) and (-) orientation in the submitted sequence. However, the sequences of some of the individual SREs share extreme homology. For example, in 1990, the sequence TATCCAT was initially identified as the response element amylase box 2. Currently, the sequence TATCCA is also recognized as part of the "TATCCA"osamy response element. This degree of identity has created instances where one identified response element completely overlaps another. This situation brings into question the accuracy of the identification of these as separate response elements by the PLACE database.

One instance where this occurred was in the predicted promoter region of ARP8. PLACE recognized an amylase box 2 motif located on the (+) strand 491 nucleotides upstream of the ATG translation start site, while also recognizing a "TATCCA"osamy element located on the (-) strand 490 nucleotides upstream of the ATG translation start site, overlapping the amylase box 2 element. This type of response element overlap occurred wherever an amylase box 2 motif was recognized by PLACE.

There were other response element combinations that commonly overlapped in the predicted promoter sequences of interest. There were six instances, spanning four genes, where PLACE recognized the Susiba 2 motif (TGACT) as well as the WB Box motif (TTTGACY, Y=C/T) as independent response elements. In the predicted promoter sequences of ADF5 and ADF11, PLACE recognized overlapping "TATCCA"osamy

motifs and SRE motifs (TTATCC) as independent response elements. In this study, response element overlaps were not counted as single *cis*-acting elements, but rather were counted as two independent response elements.

It might also be useful to examine the DNA identified in these gene promoter regions of interest for genes being transcribed in the opposite direction. If the promoters for two genes transcribed in opposite directions overlapped, instances could occur where PLACE incorrectly recognizes potential response elements in the gene promoters of interest.

THE PRESENCE AND POSSIBLE EFFECT OF OTHER HORMONAL RESPONSE ELEMENTS

Unfortunately, the simplicity of this study cannot be overlooked and the complexity of the glucose signaling pathway must be taken into account. The mRNA repression seen in the ADF9 and ARP8 genes may result from the presence of specific glucose response elements, but could also be initiated by other hormonal response elements through signal cross talk. Recent reviews have suggested that ethylene and ABA have antagonistic and synergistic effects on glucose signaling, respectively (Leon and Sheen 2003).

To investigate the potential involvement of ethylene response elements in the glucose induced transcriptional repression seen in the previously reported RT-PCR studies, the predicted promoter regions of interest were analyzed using PLACE for EREs (Table 2.9). Previously recognized EREs were identified in the majority of the samples. Although there is nothing unique about the EREs identified in the ADF9 and the ARP8

predicted promoter sequences, the presence of these response elements may be significant in influencing gene transcription.

VALIDATING THE SIGNIFICANCE OF THE PLACE RESULTS

The results generated by the PLACE database come with no guarantee. Multiple databases of this nature contain disclaimers to the validity of their results. Although a specific response element sequence may be present, functional activity may be dependent on other variables extending beyond the location and orientation of the response element motif. These factors include, but are not limited to environmental stimuli, metabolites, and other hormone signaling events.

It should not be overlooked that the glucose signaling might also be transduced in some combinatorial fashion with other still unidentified elements. Manually aligning the predicted promoter regions of ADF9 and ARP8 genes might identify sequence identity overlaps at some level. These overlaps could potentially be unknown signaling mechanisms involved in eliciting a transcriptional response. If these overlaps exist, they too should be functionally analyzed for potential *cis*-acting regulatory elements.

Characterizing the predicted promoter regions of the ADF9 and ARP8 genes with the SREs recognized by the PLACE database is the first step in revealing the potential molecular mechanisms responsible for the glucose induced gene repression seen in the RT-PCR studies. Unfortunately, this study was only able to suggest response elements that may be responsible for such repression. Experimental identification of the functionally active response elements involved in specific stimuli signaling, such as

glucose, might provide further insight to the molecular mechanisms behind signal transduction in general.

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CHAPTER THREE

THE CLONING AND ACTIVITY ASSAYS OF THE PREDICTED PROMOTERS OF THE ARABIDOPSIS ADF9 AND ARP8 GENES

ABSTRACT

Previous functional characterization of some *cis*-acting response elements associated with sugar regulated gene expression has enabled progress to be made in understanding their transcriptional control. We have become more aware that there are multiple glucose signaling pathways which interact extensively with other regulatory networks to control gene expression. In an attempt to identify active *cis*-acting glucose response elements, the predicted promoter regions of the ADF9 and ARP8 genes were cloned into the pJD301 plant luciferase expression vector. The project's initial goal was to establish promoter activities via the maize protoplast transient expression assay system, validate their repression by glucose, and then examine key response elements after their site directed mutagenesis for functionality. However, the lack of detectable activity in both promoter constructs made it not possible to complete the analysis.

INTRODUCTION

Identifying the functionality of *cis*-acting regulatory sequences in gene promoters is challenging. Understanding how putative glucose regulatory elements modulate gene expression is not well understood and requires much more research. One approach to study the function of *cis*-acting regulatory elements located in gene promoters involved in plant signal transduction pathways has been to use protoplasts in transient expression assays. Sheen (1990) used maize leaf mesophyll protoplasts to demonstrate that the transcriptional activities of seven photosynthetic gene promoters were repressed by the photosynthetic end-products sucrose and glucose.

When properly isolated from fresh plant tissue, protoplasts retain their cell identity and most cellular functions. Using mesophyll protoplasts isolated from fresh plant leaves, aspects of active plant signaling mechanisms have been examined. Transient expression assays have demonstrated regulated gene expression in response to internal and external signals, similar to that of intact tissues and plants. The applications of protoplast transient expression systems have contributed to our understanding of the functions of *cis*-acting regulatory elements and *trans*-factors in many different signaling pathways. Coupled with genetic, genomic, and transgenic approaches, these protoplast studies have helped unravel the control mechanisms of the essential cis-acting regulatory elements and transcription factors for light, phosphate, sugar, and cell cycle regulation in maize, parsley, and tobacco protoplasts (Sheen 2001).

Recent studies indicate that the actin depolymerizing factor 9 (ADF9) and actin related protein 8 (ARP8) transcripts of *Arabidopsis thaliana* are repressed by glucose (Chapter 2). ADFs bind F-actin to alter its helical twist and bind actin monomers, G-actin, in response to diverse stimuli. This enhances actin filament turnover and actin filament assembly (Bamburg *et al.* 1999). The Arabidopsis genome contains eleven expressed ADF genes (Ruzicka *et al.* 2007). Transgenic studies using Arabidopsis ADF9 mutants suggested that ADF9, a ubiquitously expressed protein, participates in regulating plant development and gene expression (Burgos-Rivera *et al.* 2008).

The actin family consists of conventional actin and various actin-related proteins (ARPs), which share moderate sequence homology (Kandasamy *et al.* 2008). Eight ARP gene sequences have been identified in Arabidopsis (McKinney *et al.* 2002). Protein orthologs were identified in other species for the majority of the ARP gene sequences. However, ARP8 was suggested to be a novel protein that is plant specific. Arabidopsis ARP8 has a complex gene structure encoding a novel protein with distinct F-box and actin homology domains (McKinney *et al.* 2002). Studies using an ARP8 promoter–β-glucuronidase (GUS) reporter fusion and ARP8-specific antibodies suggested that Arabidopsis ARP8 is localized to the interphase nucleolus, with a likely role in nucleolar functions (Kandasamy *et al.* 2008).

The advantages of the protoplast transient expression system convinced us to use this method to test for possible glucose regulation of both the ADF9 and ARP8 gene promoters. Following this strategy, the predicted promoter regions of interest were

cloned into an expression vector as luciferase fusion proteins and promoter activity assays were done using maize mesophyll protoplasts.

MATERIALS & METHODS

AMPLIFYING THE PREDICTED GENE PROMOTER REGIONS OF INTEREST

The AGRIS predicted promoter regions of the ADF9 and ARP8 genes were PCR amplified from *A. thaliana* (Columbia) genomic DNA. The ADF9 predicted promoter region was amplified using the forward primer

ADF9-F 5'-AACTGCAGAACGGTACACGCACGGAAAAA-3', and the reverse primer ADF9-2R 5'-CATGCCATGGCATGAGTGAGCTAAGATGATGTCTTC-3'. The ARP8 predicted promoter region was amplified using the forward primer

F-ARP8 5'-AACTGCAGAACGGTAATCACCGGATCTGAG-3', and the reverse primer ARP8-2R 5'-CATGCCATGGCATGCGTCGAGTTTGCGGATTTTTG-3'. The forward primers contain a *Pst* I (CTGCAG) restriction enzyme site, while the reverse primers contain an *Nco* I (CCATGG) restriction enzyme site. These restriction enzyme sites are not found in the predicted promoter sequences of the genes of interest.

When amplifying the regions of interest, 25 ng of the aforementioned forward and reverse primer sets were used in the 50 µl PCR reaction containing 0.5 µl of Expand Long Range Polymerase (Roche), 5.0 µl of 10x Buffer 3 with 22.5 mM $MgCl_2$ (Roche),

2.5 µl of 2.5 mM DNTPs, and 400 ng of gDNA. An annealing temperature of 55.0˚ C was held for 45 seconds, and an extension temperature of 68.0˚ C was held for 90 seconds over the 35 cycles of the PCR reaction.

ASSEMBLING THE PJD301 LUCIFERASE EXPRESSION VECTOR

The pJD301 luciferase expression vector (~5.2 kb) contains the cauliflower mosaic virus (CaMV) 35S promoter 5' of the luciferase ATG translation start site (Luehrsen and Walbot 1993). The CaMV promoter sequence (~700 bp) was removed by digesting the vector with the *Pst* I and *Nco* I restriction enzymes. The DNA of the digested vector construct (~4.5 kb) as well as the amplified promoter inserts (ARP8 = 579 bp; ADF9 = 1044 bp) were gel purified and electro-eluted. The amplified ADF9 and ARP8 predicted promoter inserts were cloned into the digested pJD301 expression vector using the *Pst* I and *Nco* I restriction enzyme sites designed into the amplification primers. Ligation products were transformed into DH5α competent *E. coli* cells and plated on agar LB plates with ampicillin (Appendix B). Colonies were picked and grown in 3 ml cultures. DNA was isolated by mini-preps and then screened for insert presence using restriction enzyme diagnostics. Maxi-preps were performed on samples that contained the predicted promoter insert (Appendix B).

Sequencing the Vector Construct

With the assistance of the Clemson University Genomics Institute (CUGI), the cesium chloride purified plasmid DNA from all the maxi preps were sequenced using the forward primer pJD301F 5'-CTGCAAGGCGATTAAGTTGG-3' and the reverse primer pJD301R 5'-GCCTTATGCAGTTGCTCTCC-3'. These primers anneal approximately 50 bp up-stream and down-stream, respectively, to the cloned region.

Site Directed Mutagenesis

To remove a false start translation site located in the original vector constructs, primers specific for the individual constructs were designed. The 5 base pair deletion was made in the ADF9 construct using the forward primer SDM-ADF-F 5'-CATCATCTTAGCTCACTCATGGAAGACGCC-3' and the reverse primer SDM-ADF-R 5'-GGCGTCTTCCATGAGTGAGCTAAGATGATG-3'. The same deletion was made in the ARP8 construct using the forward primer SDM-ARP-F 5'-CCGCAAACTCGACGCATGGAAGACGCC-3' and the reverse primer SDM-ARP-R 5'-GGCGTCTTCCATGCGTCGAGTTTGCGG-3'.

The 25 µl PCR reaction contained 2.5 µl of 2.5 mM dNTPs, 1.25 U Pfu Turbo, 2.5 µl 10x Pfu Turbo Buffer, 25 ng DNA, and 10 ng of each of the appropriate forward and reverse primers. The 25 µl PCR reaction was then split into two 12.5 µl samples, one of which was kept on the bench top serving as a negative control, and the other put into the PCR machine. A melting temperature of 95.0˚ C was held for 30 seconds, an annealing temperature of 55.0˚ C was held for 60 seconds, and an extension temperature

of 68.0˚ C was held for 10 minutes (2 min/kb of plasmid) over the 18 cycles of the PCR reaction.

After the completion of the PCR reaction, the test tubes were cooled to room temperature. At this point, the negative controls were treated similar to the experimental test tubes. Added to each of the PCR product test tubes was 0.5 µl of *Dpn* I. After incubating the samples overnight at 37˚ C, 4 µl of each sample were transformed into DH5α competent *E. coli* cells and plated on agar LB plates with ampicillin. Colonies were picked, grown in 3 ml cultures, maxi-prepped, and screened for the deletion.

PROTOPLAST ISOLATION

Maize plants were grown 8-9 days in constant dark, then were subject to greening by overnight illumination. The digestion medium (Appendix B) with RS and R10 cellulases was placed on the 55˚ C heat block for 10 min and then cooled to room temperature while mixing on a flopper. BSA (0.1% w/v) was added to the digestion medium after equilibrating to room temperature. Second leaves were then collected from greening plants and cut into thin strips < 1 mm wide. The tissue was vacuum infiltrated in the digestion medium for 20 minutes, then incubated for 2.5 hours at room temperature with gentle shaking. After the incubation, the plant material was filtered through moistened Miracloth, washed with 7.5 ml of wash solution (Appendix B), centrifuged for 2 min at 1000 rpm, and the supernatant was removed. The pellet was resuspended in 10 ml wash solution and left on ice for 20 min. The protoplasts were counted with a

haemocytometer, re-centrifuged, and resuspended in wash solution to give 200,000 protoplasts per 100 µl.

PROTOPLAST TRANSFECTION

Ten µg of plasmid DNA was added to 100 µl of resuspended protoplasts, in 2 ml microfuge tubes. An equal volume of PEG solution (Appendix B) was then added to the tubes, and the samples were left at room temperature for 7 min. To stop the transfection, 600 µl of wash solution was added. The samples were then inverted, centrifuged at 1000 rpm at room temperature for 2 min, the supernatant was removed, and the pellet was resuspended in 100 µl of incubation solution (Appendix B). The resuspended protoplasts were then transferred into culture dishes that were previously coated with 5% calf serum and contained 400 µl of incubation solution. Protoplasts were harvested at appropriate times by collecting into microfuge tubes and centrifuging at 1000 rpm at room temperature for 3 minutes. After removing the supernatant, samples were stored at -20° C until processing for activity assays. A pJD301 luciferase expression vector construct containing a pea *RBCS* gene promoter was used as a positive experimental control (Schaffner and Sheen 1991).

Luciferase Activity Assays

Prior to use, the Monolight 3000 (PharMingen) was allowed to warm up for 10 min and the injector was thoroughly rinsed with dH_20. The protoplasts were lysed in 100 µl of lysis buffer (Appendix B) by vortexing at max speed for 30 sec. The lysed protoplasts were then incubated on ice for 5 min. For luciferase activity assays, lysed protoplast volumes of 25 µl and 50 µl were added to 100 µl of assay reagent (Promega #E1483). The blank values are luminometer readings of protoplast samples that were not transfected with plasmid DNA.

RESULTS

PROMOTER CLONING INTO PJD3101

The PCR reactions produced fragments of 579 bp and 1,044 bp, equal to the ADF9 and ARP8 predicted promoter sequences, respectively (Figure 3.1). When assembled with the cauliflower mosaic virus 35S:CaMV promoter, the pJD301 luciferase expression vector is approximately 5.2 kb. Removing the CaMV promoter region resulted in an 'open' linear vector that is approximately 4.5 kb (Figure 3.2). When assembled, the ADF9 luciferase expression vector construct is approximately 5.0 kb (Figure 3.3 A). The ARP8 luciferase construct is approximately 5.5 kb (Figure 3.3 C). Restriction enzyme diagnostics, using the *Pst* I and *Nco* I restriction enzyme cloning sites, verified the presence of the ADF9 (Figure 3.3 B) and the ARP8 (Figure 3.3 D) predicted promoter region inserts in the expression vector construct.

After ligation, the vector constructs were sequenced to confirm nucleotide identities. However, sequence analysis identified a false translation start site beginning 5 bp upstream of the translation start site of the luciferase gene. It was later determined that this was the result of improper primer design for the PCR amplification. These five bp were removed using site directed mutagenesis. Re-sequencing the constructs after their deletion showed that the ADF9 and ARP8 predicted promoter regions were correctly present in the now modified constructs (Figure 3.4 and Figure 3.5).

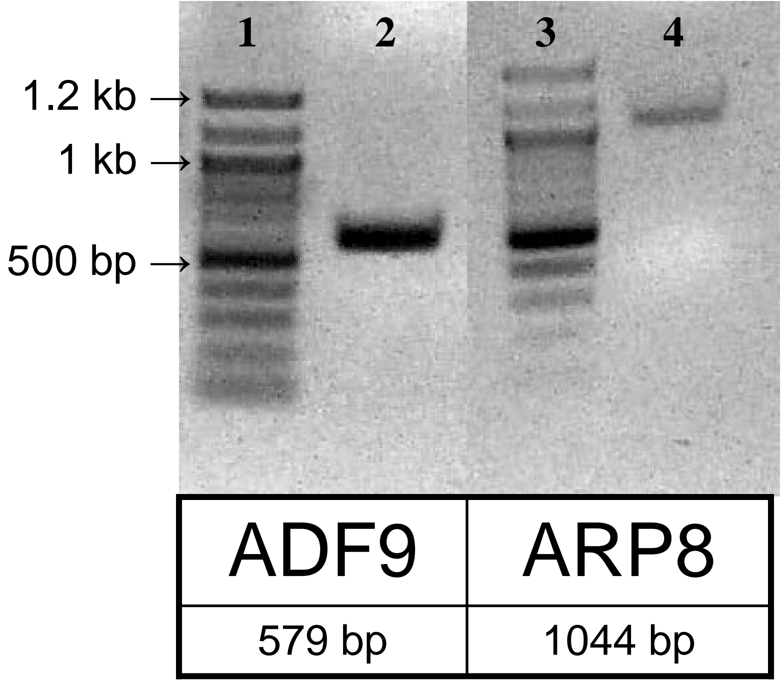

Figure 3.1: PCR amplifications of the Arabidopsis ADF9 and ARP8 predicted gene promoter regions. Lanes 1 and 3 contain the molecular ladder, while lanes 2 and 4 contain the amplified PCR products of the ADF9 and AtARP8 predicted promoter regions, respectively.

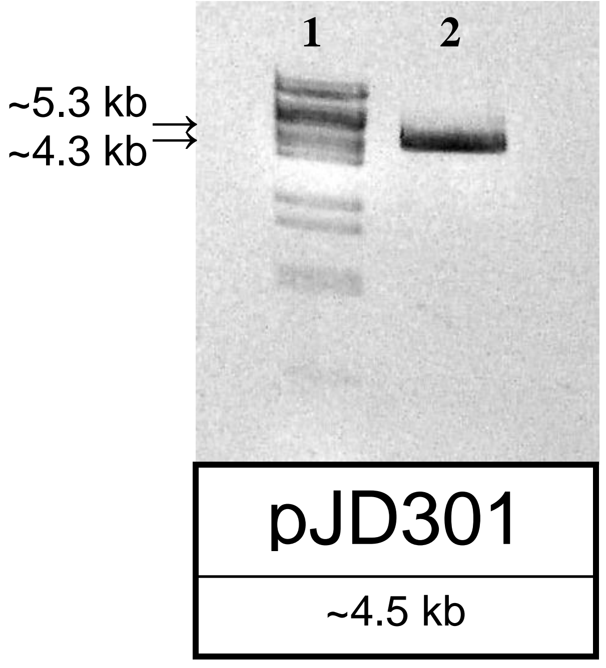

Figure 3.2: The pJD301 luciferase expression vector. After removing the CaMV promoter, the linearized expression vector construct is approximately 4.5 kb. Lane 1 contains the molecular ladder, while lane 2 contains the digested expression vector.

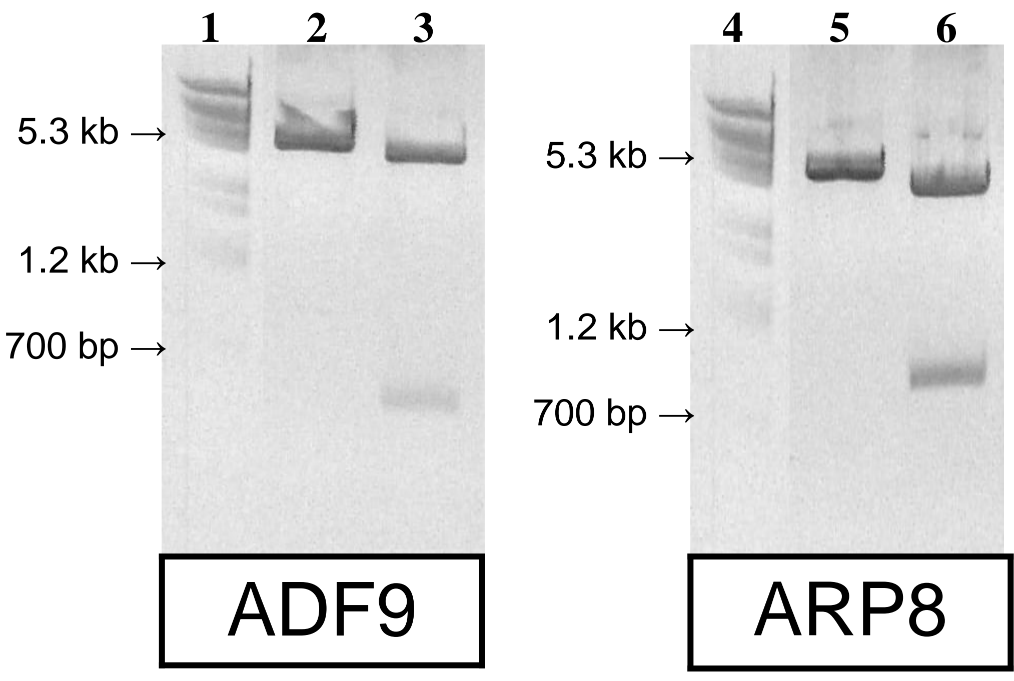

Figure 3.3: Screening the expression vector constructs for insert verification. *Pst* I and *Nco* I restriction enzyme diagnostics verified the presence of the 579 nucleotide ADF9 predicted promoter region (lane 3) and the 1044 nucleotide ARP8 predicted promoter region (lane 4) inserts in the assembled expression vector construct. Both constructs were also linearized by a single *Pst* I restriction enzyme digest (lanes 2 and 4). Lanes 1 and 4 contain the molecular ladder.

AAACGACGGCCAGTGCCAAGCTTGCATGACTGCAGAAC**GGTAC**
ACGCACGGGAAAAAACACAGCTAGCAAATTGAAACGTTAATTT
GGTAAAGAAGAAAAATATTTTATTTTTAAAAACACTTAATATA
GTTTTAATTGGTGATTTTCCCAATTCTGAACATATATATGATG
ATAAGTTTTCCATCCCAAGTTGTTTTTTCTCATTAGTCACTGG
AAACATTAATTACCAGCAATCAGTAGTTACTTTGATATTCCCC
ACACAAATTCAAATTTAAAAGAAATAATTAGTAGTACTTCTTA
TGACTGTTTTTTTTACTGAATAAAAAAGAACTATATATAGGTT
GTTCTTAACTATAACCACTAAAAAAAGGTTCTAAAATTAATTT
TAAAAAGAAAAATATATGAAGTTAACTTTTATATATTACACAT
CAAATTCCAATTTCAGGGGCTGGTAATTTGGTAATAATTAAAG
AAACTCAATGGCAATGATTTAAGCACTTCTTTATATAAGTCCA
CCAAACTACACTGATCTCCTCAACACCACACACAGCAAAACAA
AAAGCTCTCTACACTCAAAATATAACGAAAGAACAAGAAGACA
TCATCTTAGCTCACTCATGGAAGACGCCAAAAACATAAAGAAA

Figure 3.4: Final sequence of the ADF9 predicted promoter cloned into the luciferase expression vector construct. The sequence in bold font is the AGRIS predicted promoter region of the ADF9 gene. The sequence in black font is the 5' UTR region of the ADF9 protein. The sequence in gray font is the flanking pJD301 luciferase expression vector. The translation start site of the luciferase gene is underlined.

AAACGACGGCCAGTGCCAAGCTTGCATGACTGCAGAAC**GGTAA**
TCACCGGATCTGAGGATCCAATTTATACACAGCTTCTCCAAAA
AAAAATCTATGCTCCGTAAATAAAAAAAAGGTAGTTAATCATT
TTTTTCAAATACTATACACGTCAACTAATAGCAGCCAATACCT
AGTATCCACGTGTCAGATACATCATGGAATTACTTATTTTTGC
CAAATTGGTTTGGATCAGATATTAACCCAATTAACAACTAGGC
CCGGACTAATTCCCGAACATAATAAACCAATATAAATATCCGA
ATAGAATCTCGATTCCTATACTAGAATAACCAGTACCAAGTCT
GAACCAAACCGAATGGTAACCCGTAGGCCGGTCTAAAAATTTG
GAAGCCAAAGTAACAAAATACTTTTTCTAAGGTTATTATAAAC
GGTTTTGAAATTGAAAGTTTTTTTCGTAGAACTAAATATTTTT
GGGAACCATATATAATTGAACCAAATCGAAACCGAAAATTAAA
ATCTCTTAACAAAACCAAAATCCCAAGACCCAAAATCCGAATC
TGAATGGATACGATCCAAAAATGATCCAAGTAACCAAATGATC
AGAGGCCTAGTAACAACACTTTTAAAGAGGAACCAAATGATCA
GGCCTGGTAACAAAACACTCTTAAAAAGGAAGAGGACTGCTCT
CACAACAAAATAACAAAATTTGGAGTCGACCAGATTAAGAAAA
ATCCACTAAAAACAATACTTATTGGTATTTTTCTGTTTCTATG
AAATTTTGTAATTCACAACAACAACAATAAAAGTTGAGCACGT
GATATTGACCACTTCGAGTTTTTTTTTTCCAATTGGGCTCACA
AAATATCGTTATGATTATTGACATTAGTGAGCCAGTACCAAAA
TTCTTCAACGCTATTGAACACGTGTCATTATATTAAACAGTAC
ACGTCAGTCAGTCTCATCCCAACTAGAAACGACGTCACACCAA
AGAAAAAACGCCGTGTTTATCAAATTTTCCTGATTCGATTTTC
TTGGGAAAATTTCTGATTTCGAGAAAAAACAAAAATCCGCAAA
CTCGACGC**ATG**GAAGACGCCAAAAACATAAAGAAA

Figure 3.5: Final sequence of the ARP8 predicted promoter cloned into the luciferase expression vector construct. The sequence in bold font is the AGRIS predicted promoter region of the ARP8 gene. The sequence in black font is the 5' UTR region of the ADF9 protein. The sequence in gray font is the flanking pJD301 luciferase expression vector. The translation start site of the luciferase gene is underlined.

PROMOTER DRIVEN GENE ACTIVITY ASSAYS

The pJD301 expression vector has been previously used to study the effects of promoters, 5´ and 3´ UTRs, and introns on gene expression in plant cells (Luehrsen and Walbot 1993). The light emitted from the luciferase reaction is measured by a high-sensitivity, low-noise photomultiplier. The luminometer reports this measurement in terms of relative light units (RLU).

Protoplasts were harvested after 6, 12, and 24 hour incubation under constant low light. Activity assays showed minimal luciferase activity for either the ADF9 or the ARP8 constructs, while the RBCS control construct had relatively high values of luciferase activity. In an attempt to establish promoter driven luciferase gene activity, different experimental conditions were tested using the ADF9 and ARP8 constructs. Unfortunately, protoplasts transfected with increased amounts of cDNA (up to 10 fold) or protoplasts incubated in the dark did not express promoter driven gene activity for either the ADF9 or ARP8 constructs (data not shown).

Table 3.1: Average RLU luciferase values for the ADF9, ARP8, and RBCS promoter constructs in transient expression assays. Standard deviation values correspond to four data replicates. When compared to the RBCS positive control, the RLU values for the ADF9 and ARP8 samples suggests these constructs are unable to generate promoter driven gene activity.

Samples	6 hours	12 hours	24 hours
ADF9	293 ± 53	365 ± 143	628 ± 15
ARP8	214 ± 31	265 ± 33	299 ± 18
RBCS	5,126	11,652	60,766
Blank	167	217	247

DISCUSSION

Our RT-PCR data showed that glucose repressed expression of the ADF9 and ARP8 genes in Arabidopsis seedlings (Figure 2.1). To identify possible sugar response elements responsible for the signaling of this repression, the predicted promoter regions of these genes were first identified using the AGRIS database, then amplified from Arabidopsis genomic DNA, and ultimately cloned into the pJD301 luciferase expression vector. Sequencing the cloned constructs revealed that the predicted promoter regions of the ADF9 and ARP8 genes were inserted with proper orientation (Figure 3.4 and Figure 3.5 respectively).

Using these constructs, we tested for possible promoter activities in maize protoplast transfection expression assays. Unfortunately, we were not able to generate significant luciferase activity using either promoter construct for ADF9 or ARP8 (Table 3.1). In an attempt to establish initial promoter driven luciferase gene activity, different experimental conditions were tested using the ADF9 and ARP8 promoter constructs. However, increasing the quantity of plasmid DNA used for transfecting protoplasts and increasing the protoplast incubation period displayed similar results (data not shown).

The project's initial goal was to establish promoter activities, validate their repression by glucose, and then functionally analyze key sugar response elements by site directed mutagenesis. However, in this system these vector constructs failed to display significant levels of luciferase activity. Therefore, we did not make promoter mutations

that might otherwise have further defined functions of specific *cis*-acting regulatory elements.

It has been difficult to identify why these constructs are not expressing promoter driven gene activity. Perhaps the predicted promoter region we identified is not correct. If our AGRIS defined promoter region lacked an essential transcription initiation binding site found in the gene's actual promoter region, our constructs would fail to generate any promoter driven gene activity. Further studies shall reveal if the AGRIS annotation for these genes are correct.

Our constructs would also fail to demonstrate promoter activity if some of the transcriptional machinery required for this specific form of gene activity were missing in maize protoplasts, a monocot system. Our RT-PCR studies demonstrate that glucose repressed expression of the ADF9 and ARP8 genes in Arabidopsis, a dicot species. If the signaling mechanisms required for transducing this specific message of translation initiation are missing in the monocot species, then our constructs would fail to generate initial promoter driven luciferase activity. Transfecting pea and/or Arabidopsis protoplasts with the ADF9 and ARP vector constructs might help resolve this issue of lack of detectable promoter activity.

Other studies with similar research interests have used a variety of techniques to provide insight to the role of *cis*-acting response elements and to the complexity of the signal transduction pathways in plants. The effects of SUSIBA 2, a transcription factor involved in sugar-mediated regulation of starch synthesis, were blocked using antisense oligodeoxynucleotide (ODN) inhibition in sugar treated barley leaves (Sun et al. 2005).

In the α -amylase promoter Amy32b, five elements, including the amylase box 1 response elements, are essential for a high levels of GA induced expression in rice. Transient expression of the transcriptional repressor OsWRKY71 by particle bombardment represses GA-induced activity of the Amy32b promoter (Zhang et al. 2004).

As we move further away from the initial idea that plants process glucose signals through a single transduction pathway, we have become more aware that there are multiple glucose pathways which interact extensively with other regulatory networks. Identifying the functionally active sugar response elements in the predicted promoter regions of genes that undergo glucose induced transcriptional repression will lead us closer to understanding the signal transduction process at the molecular level.

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APPENDICES

APPENDIX A

THE AGRIS PREDICTED GENE PROMOTER REGIONS OF THE ADF AND ARP GENE FAMILIES

Table A.1: The promoter prediction results generated by AGRIS

Gene	Locus ID	AGRIS Predicted Promoter Location	Promoter Size (bp)
ADF1	At3g46010	Chr-3: 1692394-16921803	1691
ADF2	At3g46000	Chr-3: 16920662-16919978	684
ADF3	At5g59880	Chr-5: 24137456-24134454	3002
ADF4	At5g59890	Chr-5: 24139652-24138852	800
ADF5	At2g16700	Chr-2: 7251703-7248795	2908
ADF6	At2g31200	Chr-2: 13301129-13300756	373
ADF7	At4g25590	Chr-4: 13060132-13059637	495
ADF8	At4g00680	Chr-4: 281644-280697	947
ADF9	At4g34970	Chr-4: 16653904-16653209	695
ADF10	At5g52360	Chr-5: 21279568-21276566	3002
ADF11	At1g01750	Chr-1: 275365-274237	1128
ARP2	At3g27000	Chr-3: 9960118-9957176	2942
ARP3	At1g13180	Chr-1: 4495024-4492387	2637
ARP4	At1g18450	Chr-1: 6348099-6347901	198
ARP5	At3g12380	Chr-3: 3942265-3941905	360
ARP6	At4g28520	Chr-4: 14087572-14086840	732
ARP7	At3g60830	Chr-3: 22485048-22484902	146
ARP8	At5g56180	Chr-5: 22759256-22758240	1016
ARP9	At5g43500	Chr-5: 17486902-17486092	810
ACT2	At3g18780	Chr-3: 6474876-6472537	2339
ACT7	At5g09810	Chr-5: 3052166-3049806	2360
RGAP1	At4g35750	Chr-4: 16944867-16941865	3002
FIMBRIN	At4g26700	Chr-4: 13463661-13460671	2990
VLN1	At4g30160	Chr-4: 14753562-14751529	2033
VLN2	At2g41740	Chr-2: 17426956-17424665	2291
VLN3	At3g57410	Chr-3: 21263580-21262075	2793

The AtADF Gene Family

The AGRIS predicted promoter region for ADF1 (At3g46010)

tcaggcatctcacactcactgttgtcatcatgtagatttgtctacaaaattgatctaagatcatgagttcc
atgttacaaactctaaagctgtatgcaatttgtagtctagtgactagactctagtctagtttgtagaaaaa
tctttgacactgtctgtactcttgagctgattctgggcctcttttcttaaaataacattattgggcctatt
gagttaacgggcttcaatgttatatgcggccaggctctagagtcacatgccggtaggaaacctgtcgcaca
aagcaaactcctaactctccacgtgtctataaacgattggttgtaaccagcgtcactcagctggttcccat
caccgctataatcaaagtttcagcggaaaattggagctaaaagagccccattgattcatgtacctttcatt
tagtttcgatatttcaaaagataattagcatgttcttttagttcacgctgcggcagcggtagcaacggtaa
cgtctgagtaaaacgacgtattatgaaaaataacaatttaaaacgacaccaaaacttgaaaataacagatt
tagatgattcgctgatgacggacgatgagtcgcaacagcatcaaatgagtcgctcaaatttaacagcggca
ccttaatcttcatcgatgttcatcggctgtgacagtttttctcatttcttctttctgcataattgatttt
cttcttttctcccgttgccgctgcgttaaacaaaagatcgtggctaataaatatattcgacgccaatctta
gtggcatttctttttctatgtaggaaaagaatagtattatttccctttttctttgttacattaaggacata
ccaactagaatattaaaaatttgaaaccgatacatgaggtaccaagttcattcaagatactcctatatgtt
aataaattatgaagagtggccatcttcttaaatcgtttagacaacaatatttttgaaaacggtcaattaaa
gaagaacccaaaaccttgcgaactattttataatgtagtttatggtcaactgcaaagtctgcaatgcataa
ttatgcagtagattatgatttagaacaaaataacaataattactacctttgacccaaaaaaaattaacaa
taattttatattttaattttgtacatttttgagtgatttgctttttgaacttgtgatgttaaaaatatatt
ataaaatagatatgtgttttaccatttatttaatcagtatttgaatttttttcctatcatcgtttttcata
aatcttagttatcttttttttggagacgccgaacttattgtgaaaatgaagtatatttttttgtagttgttg
tggtgttacacatcaatatttttttacttcttaaaagttgaatcaagttctcaaaccaacaaacttttatc
gaatcaatggtacgacgaaattcaaaaatgttattacttactaattttatagatagtttaaaaatataaat
taaaaattcagtttatttatataacaagaaaaatcagtgttattattgaaacacctaccaaaataatgaat
caaaaacataaaattaattatagtattgaattcataattcaacaatattattgaaaatttgaaatacatac
caaataataatagttttttttcgtctctataatatggtggactcccgaataaagttga**atg**

The AGRIS predicted promoter region for ADF2 (At3g46000)

aagtaaaaaccaaagtttattatcatcatccccttgaaagatctattctcttattctgttatttttgagtg
atggtactggtacttgattatgtttttacattgtgttgtgaagttatgaacctataaacaacttcattcta
tcggtgtttcggtcttgtgttcgatgttgtctttggttttaaaatggtgtgtaaggaccccaaatcgtcta
ttgaagatgaacccttttaggtatatcattgatgaaatggatgattaaaagtttctctcacatttgtatga
aacaactgtctataggacaagattgatcttcagtttttttatttggtccactgattatgattggattggtc
gttttgaccattcattcttatgtttgtttccaaagaaattgcttgtgttgaccacatttgtatagccccaa
catgaagtggtaccatatcatttaataaacacagatcatgattacactatttctaaacttgtgaacttatc
caccaagttatatcacctaatccatgacattttcaatataggttgggcaatcaaataaattcttatattat
gatgattaacagaaataaaaatttaactttgggaaatagtcttatccatatattattataataaaccaaaa
tgtatagaaacatataaacaaatgaaattcgtttcattcatataa**atg**

The AGRIS predicted promoter region for ADF3 (At5g59880)

tgacttagtttggttttatagaacaaaaataacaaaattaagtattaaatcaaattattcggtttaattat
atagtcaaagtttgaaaaaatcataataatttaagagtttaagtatatatcaaccctaatttagttgaaat
tttttcaaactaatggtggttgtgttgaactggattggttatgttgccgaatataaagtgagatttgattt
aaaatggattagaagagttatcatcttaaaaactaagacgaagttaattttgagacgaagttcatgaacat
tataactaaccttaatctgtttctgtttctgtttctgaagatgattacctaaaatcctaagtctcatactt
gatacttatctaaagtatactactagaaaacaaaagtttaccctttcaatgaagatagagttataaaaacg
ttttcacgagatatcgctttgatgtgagatagcaaagagtcttaagacagtaactcttttgagaaattcct
aaatgcctttcgagataatgatttttttggttcatttatcttagagattgaaaacaaattttactttcttg
tttgatcaattatatatgtttggttttgtgagatttgatatcaatgtcatgtagagaatcacgcattcca
agttttcaactaagaaaaatacagatgggcttgtgtccataatattctttggacttggaattcttgacact
atatctatgtttgtttttaatatctatatatacatttttgcagccattttatgaaataaatcttgtagttg
ggacttatttacaatggttgccattggattttaatatttgtttttgataattagaagaaaattcttcgaat
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aaaataaaaatatttataaatagttacatagatagttacaaattgtaaactttatatatactaccttta
aaatataaattgtctccgcggtataccgcgggtaaaaatctagtaacgcctattatggtttgaaatatgat
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taaggaagaccaaataatcagcatgaaaggtcgacaattaatgaagaaagtcattttgtagaaatgcaagc
cttttgataccagcatagcatacgggcttctaaattctaatgctcacataatgtcatttattgtttaagaa
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tcaatcttaatcctattgaaacaatggtatagaaatatgcaaatacgatcatatcaactaagatataccga
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tgttttagcgcattattgcacatttgtgtgaaataatatactattttcgggaaaatcataatcgaaataga
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ttatgatgaaaggtgagccgaaagtggagccaacctgagatgccccgcatgagagccatatgcttcttcga
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tcaaacagatttttgatttttttctaagaaattttgcatgtgaatacataactacagttaattccattca
actgttacttttctttgggtgaaacaataggttggcaacttggcatagatatcaacatcagagttttgaac
aaatctatcatcatttaagaaatgatagaaaatcaagtattgtttttcaaatcaagaaatatcaaagaaga
attctgtattaagtttagttctcatggaagccatagaatatatcagaagtttgaaacaaacagcaaatata
tggaataaaaaacatagttattggaataaatagaatagttatttttttttgtttggcataaaataaagaaa
attatttgttcaacaaattgttacagatttcacttatgtatattttgaactaattcttatttttatattaa
ttaaatcaagattttacttttgaaaaataattaatgaatttatgaaacaatctctatgtaaataaaataaa
gcaaattaaaacgctcacac**atg**

The AGRIS predicted promoter region for ADF4 (At5g59890)

gatgaaagaaatcccctgaaaatttcggttgaatcaaactttttcgtactattactcagtgatgctttttc
ctaaagtttgctgtgacggtaccaacctgaacagcttcattttatcagtttggtctttgtctttgtctttg
gtctccttttgggtccttatagtgttgtcttaaaacttaaggaatctggcttgtgctctttaccattgtg
gaagaaatcgaacccttttattaaacaagaatcctattggcacatcttgtcttcacataatgaaaactaatg
atccagggattaaaaagttgtgaaagttttataggcaagttgatactgtgcaaaggtaatatacattttca
aacataaggatctctggattgatagaatttcatttcgtacacgactattcacatattcgtcaatgcgacta
tttcaggttcaaagtattaggaatttaggatagttccaataacgagaagagttgctcaatcgagtttggtc
caataagataaactgaccatcaaacataaaatcctaatcgatacttttgattatttggaaacagatccaac
cattggcagttgtccattgctcattctacgcaatctttcaactgtcaaatcttagcccaacaaaaatactt
agatgattattatatgttgaccaaaacaataataatatgcttaccatatagtttattaattcataaataaa
ttgaagaatacgaaattacgaagaaaataaataaaaatacaaaaagcaggttcttctctatatattcggaa
tttagcttccaaataacgc**atg**

The AGRIS predicted promoter region for ADF5 (At2g16700)

taaattggatgacgtaagaccttaaatctatatagctccaaataagtttcatttctattatatatcttcac
cactgtaacataaaccaaattttacccggttaagaaaaaaggaaaaaaaaatcttaaacattttttttgta
aactagttctacccaaaatgcacttttgagatcatattagccaataaaaggggttattgtggatttgagca
ttttttagcccaaaattgaaaatgcatttagctaatagactttgaaaatatgtacttttatagagggaaaa
tgacgaatttgtccttacttttctctggtttttttttcgatgttctttttcttcatcctttttttccccttg
aattgcttttgttcttccttcatgatttttttcttttttttttcctcacaaaatttaaatagtttttaatcag
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ccaaatattagagaaatagaacatttcattggacttatgaaaataggcatattttggtctgtaaaattaca
gttttacccttattcttcctttcttcttccttctgtgacgtcgatttcattttttttttctgtttcttccaa
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aaagaaatctaaaccctaataggatgatatatatagggaacccaaatgtttcaatcttatacacatattaa
atcagttacttatttgagaattcaattgaatcaacctcttcaattacatgtttaacaagaaacccaaacaa
agaaaatcaaagatttaataaaatctacaaacatgttgatgtgtaattttagatggataatttaattgttt
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caacaaagtttgtgaattttgtgaatgaatggttctatttttggataatgaaaattagtagaagatgagt
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ttggagctatccagacaattaactctaatcataatggtttatagatttgaaaaaaaaaaaatatagatttt
gatttaaatttttatttaatattcctgaaataaaaaaagaataaaaaaccggaaaaggattggaggtgaaagg
cttgtcgacccaacgacctcacatggacatatatttttctcacctaacattagattttaatgtttggtatt
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actctcgtacagtcgtactcaatacacaaattaatatcgattttatatataacagttataacacacacac
gtctatatatttatttacttatatttatgtacttgtgcgtatatatatctatatatggtatagtacaaatt
catgggtcgacaatatatgttgtgaagttgctcactttgtaccggtcatgatttcatatattggacaattt
catgggaagactcggatagtttcggaatttaactaaacttttgaaattgtgtagtgaccttcaacgtatcc
ttatcttatattctatttaccatttgtgcttgtactaccagtttacccctctcacatatgactataataat
aatttcttacctataactattagactattattgttttggatgccccatgtcatcgctgaattatctgtttg
aattgtcaacttatataccaataatagaagatgaaaaattaacgaaatcgaaacgagataatatgagatcg
atatctcaattcttagctttcacgcaaagggtcacttgagttttgagtaacacgaaattgtaaagatcaat
aaaattgtgggtgttggttttacttggaggatccacttctattcattgggagtttgggaccaccctctcca
ttcctagcgtcacatttttctaagtattttaattgcatataagcaagcattgacctattatttctcttctt
cgtatcctatatatacatatactttgattttctttgtaatcccataatgtgtttagtcgcgcaccctataa

tcataatggtgacgagaggttcgagaaaaaagtggtgaagagagtgccgacgagaatgtttttcaaagatc
ataactgaataactaattttatctataaaagaaaacactaatctattatgtttttttcaatggttgatcaat
tcttttgcaaatgaaaatgttgatccaaattccatacgcaaataaatcgttagatatttttaaaatatata
taaaaaaaattattagatatttatcagaaaaagacaagatttattagtgcagaacacacggtgacgttaat
atgccgacgagaaaaagaacaaaaatattcgtctctaacaaaaaagtttacaagggtgagtttgtaataag
agtaaatagtgatcgcatttgtataggcataactttttatataaagacctcaaagtcttaccatttga**atg**

The AGRIS predicted promoter region for ADF6 (At2g31200)

atctctgagacacgatggcacagtcaggttccgaacgttaagagtcgccggagttggagtgaaccaccaca
acagtaatcgcctagagccgtctaaaaacaaaatcaaagagattatcttaacaacgacgtcgtttcttcgt
tccttttctacttctgaggcccaaatgatttttgttactattcactaatgggcttaattaaggcccaatat
tgatagatacgcggcgactagttaaggggcaacaaatatggcccacttaaaaagaaacacgacttttctta
tcgcgttgttctaacgccaccgaccgaacacttctttttcttctgcaataaaccaaaaaaagttaaaaatt
caaaaacttaattactaa**atg**

The AGRIS predicted promoter region for ADF7 (At4g25590)

ttcacaatattctcacataacaacaaatctttcgatcttcctcaagtcttatcatagaacctagacaaaag
caatggtttgttctcttctctttttctaattcatttgtaggattgtttggttctagcattacatgccatca
atgtgatgatttttttctagtcggggcgaaagatacacgatattcaaatctatgaaatgtactagtcacaa
aatctgatttaatttttgtctaatcatatgttaattagacgtcgattttaccatgttattgacgtgtctct
cattttacatgctatattttgtgtcttgtctagttcataattgtcataactcaattgctagactaagatta
ttactaatgttttgacatatggaaattagtaatggaaccgaaaacgcctagctaacttgaaagtatttttt
tcccccggtatacaaaaaatgctctcctcctaatttatgtgaatgaaaaggcgaacgcggcgtcgggga**at**
g

The AGRIS predicted promoter region for ADF8 (At4g00680)

tgatacaatatattaattagaatcagtaccgcaacaagcttcttcgtttctgttgatgatactaagaaatt
gttgccatctttaaaaccccgattactagaagaatgttcctcaatcctatcatcattatgtttaacaactt
gttgagattgtgaacaccttgctgctttctttttaatgacactgaagaagcacgaaccaacaatttgaac
ctgagaaatcgtttgcaactggcgagaagtctttgtctgaaactcgatgttctagtacaagacgaagagac
tggtttgtccgagatggtgcgattgcgaatcttgggtacaagagccattagagatcgacttctcaggaaaa
aaatattttagggtttaaacataactttcttctacttcacccactcgagagaaacgtcaatgttggaaatt
gaatctttgcttaatcgtgtacaagtgaaatcagaagagaaaggtgagaaaatgaagtgtagagagatgtg
tgtttaaggaagaagaagaggcgcgctcgagtgtagaagaacgaagaagaagaagaatagtagtgaaggaa
aagagagaaaaaggccattgggccagaagtgaacaaggcccaattatgggttcaatggtcgtttgggccgc
ataaaacaatatttagcatagaggctaaagtcaaagtatatagaagagaaaaaaatatatataacatgttc
atcatgaaaacttcaacggtccaatgatctcccgaccaaagttgagctcatatgcaacaaacacaaataaa
tatagatttatatgtattatcaaggattaaaactatatataggtttgaaaaaaatgggcacgacgtgatag
catcaaagtcatctatttcaagacgtgatcagcctaaagaccaaaagaaaatatcaaatattacccaactt
ttgtgtttttcctcaacctcataa**atg**

The AGRIS predicted promoter region for ADF9 (At4g34970)

ggtacacgcacgggaaaaaacacagctagcaaattgaaacgttaatttggtaaagaagaaaaatattttat
ttttaaaaacacttaatatagttttaattggtgattttcccaattctgaacatatatatgatgataagttt
tccatcccaagttgtttttctcattagtcactggaaacattaattaccagcaatcagtagttactttggt
attccccacacaaattcaaatttaaaagaaataattagtagtacttcttatgactgtttttttactgaat
aaaaaagaactatatataggttgttcttaactataaccactaaaaaaaggttctaaaattaattttaaaaa
gaaaaatatatgaagttaacttttatatattacacatcaaattccaatttcaggggctggtaatttggtaa
taattaaagaaactcaatggcaatgatttaagcacttctttatataagtccaccaaactacactgatctcc
tcaacaccacacacagcaaaacaaaaagctctctacactcaaaatataacgaaagaacaagaagacatcat
cttagctcact**atg**

The AGRIS predicted promoter region for ADF10 (At5g52360)

aaacctagcatcgaaatttgtatttttttttacctctgtacacagatatttgacttatgatctgaagtgta
gttcatgttatttagagagtttaacattatattctctgaaaacgtatttgaggctatactgtcacttgttt
gtttgtttgttaagagtagtcactaagattagatttggttgctgtattgattcttttgtaggagggaccaa
atttgcaagttgtttcatatgcaagggacaagggcacataagcaagaactgtcccgaaaacaagcatggaa
tctacccaatggtacaatgctcaaatcttgtttcgagcctttttcttttcctcatagaagatgagttttgat
atgttgaacaatggatttggtgggccttcttttgcagggcgggtgttgtaaagtgtgcgggagtgtggcgc
atctcgtcaaagactgtcctgataaattcaaccaagagtcagcccaaccaaagaagacttcaagtaacatt
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acaggatttgatgctacaccaagaggaaagctcactaagtttagtggggacgatcttgaagacgatttcac
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cacaaacggtgttggaagtatatcccaaacctgtcaaagactgctcctgctacaacctctgctcatgtcca
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gttgttgtgacttgatagtttctttgaattcgttgatagctttgtaggaaatctacggatgatgatctgtt
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agtttctatcttccagaatttggtgaatcctaaaccaaaattccaaattttcaatatttgaaagtctccat
gttatatgcaatggaagatagtcttgttggaccaatttaacatttcttccaaccataacgaaaaaagcata
gttgtacccctttttccttggactgaattgcatcacagactccaaaaccatcgttttgattcacctttaat
gaaagcagtttttggttgaaccaatgttagtaaagtattattaggaatccacatatgaaccaatccagtat
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ggttctattcaatagctttgtggttaacaaagaaccaattttaaaatctctattgaaccagtttgaatcca
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aggtccaaagattgtcaatttcgttggatgatcacaagaggatcgaaaagggtcatcaatttttgtaggat

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aacaatgctatgtaatggaaccacggccaattctgttacagataatcttctaaaccgaataaaacccacct
agaaccaaattataacatatagtccttgataaggatgtaagaaatgctaaaattagtgtgaaaaacgtccg
gaaagaaaggatatggttaggcgaatttgaaggatagagagtaaaaaaaggtgtggtttgatgttataaga
tggagaggtattaatggttgtccgagttcgtctccattggcctcttcaaatcaaattaagaatctcttatt
cccccaaatttttcatttct**atg**

The AGRIS predicted promoter region for ADF11 (At1g01750)

atggatgcgttgcttcaaattttcaattataaggtaactggggtaaatatgtctggaaacaaggagaaatt
tcccaatcttactacttacagagagatatcacgcagagatcaaaattgaaaatggggtacagaaataggtc
gtaccttgtcaagtcgctaccattctagtgagccaactcggcggctagtgacagcgagaccagttactaat
ttggaccagatgaaagaaagaagcaaaacacagctgaactaggaagccttcttatctagtcatctagatac
acatttgccgccggtattcgtatcgatgggattctatttctcgcactagctagtagctaaatccacgaagt
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cgatgttgaagtagccaaagcagaccgggaagtgtcgctagacaaatagtataccgacagagtataaatat
tatggttggacttggagaaggcaaacaaagaatctaaagccgccacaaattttctcaatttaattgatcac
gtacaaaaaatattggggttttatgtaagcgagtgtgtctgtctttcggtgggataagcccatgggcttat
gttaggccctcattgaccaacaatttttttctgtttggtcctcaattaaccaaaacaatagaaaacgtttt
tgtgagtccaaaggttttattttctcgacatgacactgatacaattggttgcttattttgtccaaatccat
tctttggataatatgatgatcaatatctgcagtttatcatagcgcaaaacccgtttgaagagaaatgacga
aatcgaaggctaattattgtttttttcgctaaattctaattaaatccgcacccaatagattagcaaagtct
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acctcaactccccaatgatgtccggaacaaacttcaccacatgtgcttgcatatacacatagatacatgtg
tattatgcgaatgcgtgaataactgaatataatatagactcactctaatatatctaatgaagt**atg**

The AtARP Gene Family

The AGRIS predicted promoter region for ARP2 (At3g27000)

aaaaaaaaaactcgatacctttttgttttacaaagtcgtcacaattctgaaacccttaattgaatccaagag
aatttttgattaaattaagtctctggaccctgaagtccccacacaagcaagcttcgatggatcccaagaac
ctaaatcgtcaccaagtaccaaatttcttgaacccaccaccaccaccgcgaaatcagggtttggtagatga
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aagctgagccatcgattattgcagctactggttcaggaactataccggcctctgctttagcttcttcagct
gcaacctctaaccatcatcaaggtgggtctcttactgctggtttaatgatcagtcatgacttagatggtgg
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caaagataagtataaattgttcaacgagtcaaaaagtttatatcattttactcattttgacttgaatttga
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tgagcaatgatctaactcaacactatggtccaaatacaactccaatagatccattccaaagcctccctcat
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aatagtgattgaaatctctgatcaagggattgcatgatgatcatttactaattcttagtttagatcatcta
gaagattgttatacaagtaaacagaattatctgtttgacctaatatgggcctgagatagcccatcaatacc
cacaaccaagttaactccatctcaataattcgagaaaaggggtaacgtatttaataatttttttctatgtat
tgaccgtttgcactacgtgacgttttcacgtagagtaaacgacgcgcattcttataaagaaacgaggaaac
agctgctttttgtttgtcgaagagcaaatat**atg**

The AGRIS predicted promoter region for ARP3 (At1g13180)

gatccggtgaaggagatcgaagaaatgttgagtttgaactagaaaaaaaaagagaagcagagagagcagca
atggagggctcacagatccaatttaaggcagttgtcagtaaaattttttgtgtttgtgcagtgtgcagag
actctgcatagagaagagagagagatagagagagagggagcatctctctgaaatcgtttaaaattaaatca
tgaaaatagaaataaaaaaggtaattatttttactaataatgctctgagtcaacataattggtgttttcta
tggaccaatcctactagagcatagattatatgtatcgaatttccatatttgttttgaaacataaaaatatt
taccaaattgttactgtctttttttatttttttgttggtctcaataaccaaaatattcggtaccaatactcaa
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tataatactgctaatacattgatgagtatttataagtaaaaaaacttgtagaatcttaaaaatatatttag
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tcaacaatgagtcgtctggaaaacatgtcggcaagagatgcttacagcaatgtgactgatctaaaaacgat
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aacgaatttagtaatttgttggacgaatgtagtaattcgttagatgaatgagatcaatcaagccatttaaa
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ccagtatggttcaattcaatgttattgaaatttgtttggtttttaaatcagacttggttattgagtcgggt
tggaaaaccaaattaaccacagcgcataaggacgaagccaaaatttaaaacagtagttgtacataacttca
tttgtttttt**atg**

The AGRIS predicted promoter region for ARP4 (At1g18450)

gcatagacattttatttggcaataaatcagcttcgtttactgcaaaaagggtggtcacaaaagttggttcg
gtttagaaaaattgtgttcggttcggttagtttcttgaataatttaaacccgacggattaaatgagaggtt
ttgtggtccccacaactcgaactcagtaaaaccaagtcgacccgattcagctggat**atg**

The AGRIS predicted promoter region for ARP5 (At3g12380)

ttgttaggttggagaagaccgagtgttaattattgcaaggaaaatcaaaaggcccaaaacttttatataaa
atcgcaatattacacattgtccgttgggcttatcagctggaaaaaattggtcggaatatttcagttaaaga
aaagcagcaaaaaagacaacctatgtcggaatacgaaaacgccgtctgtggggatcgaacccacggccacg
tggttaaaagccacgcgctctaccactgagctaagacggcttacgtttatgtcaacgacattaaactattt
attcaattatcaagtctttttaagtcagagggttttgggcctaataatgtttttccacgtcacggagctc
cattt**atg**

The AGRIS predicted promoter region for ARP6 (At4g28520)

ctattggagtaaatgggacggtgtcacattttccgttttggaaatgaactttgggctcacgttatgggcta
ttagatatttgatgggctttctagtaaatacaatataagttattgggcttagtttaaataagcccatgttg
gaaatatttgacacatgtcttggctactagtgctaaacatgcaaccgaacagttgtcgagacaagtcgcag
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ggagagtgagctacgcgcatgaagatcaagagacacttgctcgagctcacagagtgacgtgtaaaaagctt
agactgaagtccccatgcaaacctaatcctacgtggctcaaaccacgagctcacttgacaatatataaact
cctcctaagtcccgttctcttc**atg**

The AGRIS predicted promoter region for ARP7 (At3g60830)

ctggctttgttaacaagaaaaagacggagactcaaaacaataccgttttggacgtggaaattacgggctgt
aataggctttataatgggccttatttgggctttgaattccaaagaaataatctttcttttcttttcttctt
cttc**atg**

The AGRIS predicted promoter region for ARP8 (At5g56180)

ggtaatcaccggatctgaggatccaatttatacacagcttctccaaaaaaaaatctatgctccgtaaataa
aaaaaaggtagttaatcatttttttcaaatactatacacgtcaactaatagcagccaatacctagtatcca
cgtgtcagatacatcatggaattacttattttgccaaattggtttggatcagatattaacccaattaaca
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gccagtaccaaaattcttcaacgctattgaacacgtgtcattatattaaacagtacacgtcagtcagtctc
atcccaactagaaacgacgtcacaccaaagaaaaaacgccgtgtttatcaaatttcctgattcgattttc
ttgggaaaatttctgatttcgagaaaaaacaaaaatccgcaaactcgacg**atg**

The AGRIS predicted promoter region for ARP9 (At5g43500)

attggaggagttcggggtgcacctaatgttattccattttccacagatggtacacaagcactcagcttctg
taacttgtgtggcgtggttccattttctgcgaaagaattaaagactaatgttacaacaaacagttaataaa
ctacattgttaacatttcagaccaaaaccaagaaatacggatcttgcatcattactccaaaccaacaaact
accacaccactcagatacccataaatttaaaaaatcttttaaggtaagaaaacatatcatgattggatgaa
actattagatgcttctaagtaccaaaccatatatcatacgatggttaatatggcaactacactcctcattc
accagacagagaattggatcagagaataactcaccatacaggaatccttatcggtttatttatctcatcgc
acatttgaaatcaagaatcgttaaatataaaatacgaagataagtatccacatatataccaaatcactaca
ttacttttgaagtttttatagaaaaaaatctcggaagaaattttatcttatctgtttatttgttctcacc
gcacttttcaaatcaggaatcgttagtatcaacacaaataccaaaatcactacattagttttgagtctcat
tatagaagaagatcttgaaagcaattttatcttatatatcctactttttccactatactttaaaacggaat
cacaactttgcgaaacatatcgctttcgctggtttcagttgccagcgataggcaaacgaacataatcataa
acttaagattctaacaaagtaaagttttc**atg**

APPENDIX B

PROTOCOLS

RT-PCR PROTOCOL

RT-PCR Reagent Protocol

*dH_2O	1.0 µl
*cDNA	2.0 µl
*2x Taq Master Mix	5.0 µl
Forward Primer (10 ng/µl)	1.0 µl
Reverse Primer (10 ng/µl)	1.0 µl
	10.0 µl RXN

* indicates materials used in the master mix

RT-PCR Cycle Settings

Step 1 95.0˚ C for 2 min
Step 2 94.0˚ C for 30 sec
Step 3 55.0˚ C for 30 sec
Step 4 68.0˚ C for 50 sec
Step 5 Go to step 2 34x
Step 6 68.0˚ C for 7 min
Step 7 Hold at 4.0˚ C for ∞

CLONING PROTOCOL

Following the Roche Expand Long Range Polymerase PCR Amplification Protocol, the DNA fragments of interest were amplified. 10 µl of the 50 µl PCR amplification reaction were run on a 1.5% agarose gel to determine if the reaction was successful. The remaining 40 µl of the PCR amplification reaction were digested with *Pst* I and *Nco* I to produce clean fragment ends prior to construct assembly. Following the restriction enzyme digestion, the DNA was ethanol precipitated and gel purified. The DNA was electro-eluted from the gel block, precipitated, and an aliquot was run on a 1.5% agarose gel to validate the presence of the fragment of interest. 3 µg of the re-suspended precipitation product was ligated to 1 µg of linearized pJD301 expression vector. After an overnight incubation at 16ºC, the ligation product was transformed into DH5α competent cells and spread on +amp selection plates. After 16 hours at 37ºC, colonies were screened for insert verification using PCR, and mini-prepped DNA was screened using restriction enzyme diagnostics. Colonies containing the insert were processed for maxi-prep plasmid DNA extraction.

Roche Expand Long Range Polymerase PCR Amplification Protocol

*dH_2O	35.0 µl
*10x Buffer 3 (with 22.5 mM $MgCl_2$)	5.0 µl
*2.5 mM DNTP	2.5 µl
*gDNA (200 µg/µL)	2.0 µl
Forward Primer (10 ng/µl)	2.5 µl
Reverse Primer (10 ng/µl)	2.5 µl
Expand Long Range Polymerase	0.5 µl
	50.0 µl RXN

* indicates materials used in the master mix

PCR Amplification Cycle Settings

Step 1 94.0˚ C for 2 min
Step 2 94.0˚ C for 30 sec
Step 3 55.0˚ C for 45 sec
Step 4 68.0˚ C for 4 min
Step 5 Go to step 2 34x
Step 6 68.0˚ C for 7 min
Step 7 Hold at 4.0˚ C for ∞

Ethanol Precipitation Protocol

1. Bring volume of PCR product to 100 µl with dH_2O.
2. Add 100 µl of cold phenol/chloroform/isoamly alcohol (24/24/1).
3. Vortex briefly and microfuge for 3 min at room temperature.
4. Transfer supernatant to a new MF tube. Add 18 µl of 10 M ammonium acetate and 250 µl cold 95% EtOH to each MF tube.
5. Mix and incubate tubes at -20˚ C for 20 minutes.
6. Microfuge tubes at 4˚ C for 10 min at max speed (13.2K).
7. Remove supernatant and rinse twice with 200 µl of 70% EtOH.
8. Spin dry MF tubes in CentriVap (max speed at 30˚ C for 10 min).
9. Resuspend DNA in 30 µl of dH_2O.

E.coli Transformation Protocol

1. Add 50 µl of competent bacteria cells to 5 µl of ligation product and incubate on ice (MC1061 cells ~ 15 min, DH5α cells ~ 30 min)
2. Transfer MF tube to 37˚ C water bath, and incubate for 5 minutes
3. Add 100 µl of LB to each MF tube, and incubate on rotating drum at 37˚ C for 30 minutes
4. Spread cells onto selection plates and incubate at 37˚ C for 12-16 hours

DNA Maxi-Prep Protocol

1. Prepare Terriferic Broth (TB) media
   Autoclave:
   - 200 ml H_2O
   - 10 g TB powder
   - 1 ml glycerol

   Add 200 µl of ampicillin once the TB has reached room temperature

2. Add freshly transformed bacteria culture from growth plate to TB. Incubate at 37˚ C while shaking for 10-12 hours.

3. Centrifuge media in 1 L bottles at 4200 rpm at 4˚ C for 15 min. Remove the supernatant.

4. Resuspent pellet in 40 ml of Solution 1 (10mM EDTA, pH 8).

5. Add 80 ml Solution 2 to each sample and mix well.
   Solution 2 should be made fresh before each use:
   - 980 ml H_2O
   - 20 ml 5 n NaOH
   - 50 ml 20% SDS

6. Add 30 ml of Solution 3 to each sample and gently mix.
   Solution 3 can be stored at 4˚ C and should be used cold
   - 200 ml H_2O
   - 1 kg K-Acetate
   - 600 ml acetic acid
   - adjust volume to 4 L

7. Spin samples at 4200 rmp at 4˚ C for 5 min. Carefully decant supernatent through a wet microcloth filter and into a clean 250 ml bottle.

8. Fill the 250 ml bottle with isopropanol and invert several times. Centrifuge the sample at 4200 rpm at 4˚ C for 10 min.

9. Remove the supernatent and wash the pellet with 95% ethanol. Invert the bottle and dry the pellet at room temperature for 20-30 min.

10. Resuspent the pellet in 3.5 ml of Solution 1.

11. Transfer the solution to a 15 ml Falcon tubes containing 5.5 g of powdered CsCl. Using the flopper, thoroughly mix the solution at room temperature

12. Add 300 µl of ethidium bromide (10 mg/ml). Mix well.

13. Spin solution at 3000 rpm at room temperature for 7 minutes.

14. Transfer the supernatent to a 5.5 ml quick seal ultracentrifuge tube. Seal the tube.

15. Spin samples at 60,000 rpm at 24˚ C overnight.

16. Using a sterile 3 ml syringe and 20 gauge needle, pull the DNA band from the ultracentrifue tube and expel the solution into a clean 15 ml Falcon tube. Adjust the final volume to 2.5 ml with H_2O.

17. Add 7 ml of n-Butanol (saturated with 1 M NaCl) to the solution and shake excessively. Let the solutions separate and remove the supernantent. Repeat this process 2 more times, for a total of 3 EtBr extraction washes.

18. Fill the Falcon tube with 95% EtOH. Invert the tube and observe the precipatation of the DNA strands. Spin at 2500 rpm at room temperature for 4 min.

19. Remove the supernatent and wash the pellet with 70% EtOH.

20. Remove the supernatent. Invert the Falcon tube and dry the pellet at room temperature for 20-30 min.

21. Resuspend the DNA in TE, quantify, alloquate and store the samples at -20˚ C.

SEQUENCING PROTOCOL

ABI BigDye Terminator Sequencing Reaction Reagent Protocol

*dH_2O	4.0 µl
*sequencing primer (10ng/µl)	2.0 µl
*BigDye	1.0 µl
*5x BigDye buffer	2.0 µl
DNA (100 ng/µl)	1.0 µl
	10.0 µl RXN

* indicates materials used in the master mix

PCR Nucleotide Labeling Cycle Settings

Step 1 95.0˚ C for 5 min
Step 2 95.0˚ C for 30 sec
Step 3 50.0˚ C for 10 sec
Step 4 60.0˚ C for 4 min
Step 5 Go to step 2 50x
Step 6 Hold at 10.0˚ C for ∞

Sequencing Product Clean-up Protocol

1. Add 10 µl dH_2O
2. Add 2 µl 125 mM EDTA
3. Add 2 µl 3 M sodium acetate (pH 5.2)
4. Add 50 µl 100% ethanol
5. Mix by inverting
6. Incubate at room temperature for 20 min
7. Spin at full speed (13.2K rpm) at room temperature for 20 min
8. Remove supernatent
9. Wash non-visible pellet with 70 µl 70% ethanol
10. Spin at full speed at room temperature for 15 min
11. Invert tubes and dry at room temperature for 30 min

Protoplast Reagents

Maize Protoplast Digestion Medium

dH_2O	0.65 ml
0.8 M mannitol	3.75 ml
0.2 M MES (pH 5.7)	0.5 ml
1.0 M KCl	-
1.0 M $CaCl_2$	5.0 µl
14.2 M mercaptoethanol	1.75 µl
R10 cellulase	-
RS cellulase	75 µg
R10 macerozyme	15 µg
	5.0 ml

Protoplast Wash Solution

0.8 M Mannitol	37.5 ml
0.2 M MES pH 5.7	1.0 ml
1.0 M KCl	1.0 ml
dH_2O	10.5 ml
	50 ml

PEG

PEG	1.0 g
0.8 M Mannitol	0.75 ml
1.0 M $CaCl_2$	0.25 ml
dH_2O	0.625 ml
	2.5 ml

*Allow PEG to mix on flopper for ~ 2 hours prior to use

*PEG has a shelf life of 2 weeks

Protoplast Incubation Solution

0.8 M Mannitol	31.25 ml
1.0 M KCl	1.0 ml
0.2 M MES pH 5.7	1.0 ml
dH_2O	16.75
	50.0 ml

Lysis Buffer

1.0 M Tris-phosphate pH 7.8	1.25 ml
100 mM diaminocyclohexane tetraacetic acid (in DMSO)	1.0 ml
glycerol	5.0 ml
10% Triton X-100	5.0 ml
dH_2O	37.75 ml
	50 ml

*Add 10 µl of 1.0 M DTT for every 5 ml of lysis buffer prior to use

* Store at -20.0˚ C