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Identification and characterization of protein phosphatase 2A mutants in Arabidopsis thaliana

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IDENTIFICATION AND CHARACTERIZATION OF PROTEIN PHOSPHATASE 2A MUTANTS IN *ARABIDOPSIS THALIANA*

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To my parents, their love, support and caring, was, is and always will be greatly appreciated.

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ABSTRACT

IDENTIFICATION AND CHARACTERIZATION OF

PROTEIN PHOSPHATASE 2A MUTANTS IN *ARABIDOPSIS THALIANA*

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University of New Hampshire, September, 2008

Reversible protein phosphorylation is a highly regulated process that affects almost all cellular activities. Phosphorylation involves two groups of enzymes: protein kinases and protein phosphatases. Considering the central role of these enzymes in cells, elucidating their function is extremely important. My research focuses on protein phosphatase 2A (PP2A) C subunits. PP2A is one of the most abundant types of serine/threonine phosphatases in all eukaryotic cells. Compared with animals, PP2A function is not well known in plants. Early studies using protein phosphatase inhibitors are not useful to demonstrate the function of individual PP2A subunits. A reverse genetic approach can be helpful to gain insight into the function of PP2A in plants since a resulting phenotype is due to the lack of function of a single protein and provides direct evidence to elucidate function.

In this study, 13 homozygous mutant lines generated by the insertion of foreign DNA (T-DNA) in all five *Arabidopsis* PP2A C subunit genes were identified by the polymerase chain reaction. The precise location of the insertion was determined from DNA sequencing, expressed sequence tags and cDNA data. The presence of a full-length

mRNA was not detected from five of the 13 mutant alleles (*c1-1*, *c3-1*, *c3-3*, *c4-1* and *c4-2*) as determined by the reverse transcriptase-polymerase chain reaction. Wild type and mutant plants were compared under different growth conditions and chemical treatments to characterize the effect of the mutations. There was no obvious difference between mutant and wild type plants except in one case: growth in the presence of NaCl.

At greater than 50 mM supplemental NaCl, roots of *c4* mutant seedlings grown on vertically-oriented plates had a strong right-skewing growth pattern (when viewed from top of the plate) while wild type seedlings showed only a slight right skewing. Complementation confirmed that the *c4* mutant phenotype was due to loss of PP2A C4 function. The *c4* mutant phenotype did not appear when seedlings were grown in the presence of KCl, LiCl or $CaCl_2$. Additional experiments showed that Ca^{2+} and auxin transport might also be involved in the NaCl-induced skewing phenotype of the PP2A *c4* mutants.

CHAPTER I

INTRODUCTION

Arabidopsis thaliana

Arabidopsis thaliana (L.) Heynh. is a modest little flowering weed belonging to the mustard family (Brassicaceae) and is related to a number of common vegetables such as cauliflower, broccoli, cabbage and radish. Several significant features of this plant have led to its wide adoption as a model system for plant laboratory research: small size (about 30 - 40 cm in height), short life cycle (2 to 3 months for one generation), minimal care, prodigious seed production (with an average yield of 10,000 seeds per plant) and a relatively small genome size (157 Mb) compared to other higher plants (estimated to be 2,500 Mb in maize and 16,000 Mb in wheat; Patrusky, 1991; The *Arabidopsis* Genome Initiative, 2000).

The first *Arabidopsis* paper was published in 1873 by Alexander Braun in which a mutant plant was identified in a wild population (Meyerowitz, 2001). The mutation was in the *AGAMOUS* gene which encodes one of the floral ABC regulators (Yanofsky et al., 1990). In the 1940s, *Arabidopsis* was first used as a genetic model organism for laboratory research by Friedrich Laibach who described *Arabidopsis* as "the fruit fly of flowering plants" (Patrusky, 1991), but it was not until the 1980s that *Arabidopsis* became widely used in plant biology, genetics and physiology as a model plant. With the completion of the genome sequence in 2000, *Arabidopsis* now serves as an excellent

organism for sophisticated studies in genetics and genomics including full-genome array analysis, complicated phenotypic screens, etc. In addition, there are many community resources available including a variety of mutant and clone collections. This research utilizes one type of mutant collection (plants with random T-DNA insertions) which enables researchers to use reverse genetics to analyze gene function. Data obtained with *Arabidopsis* can be applied to other plants, including crop species.

Protein Phosphorylation

Protein phosphorylation is a highly-regulated and reversible process that affects virtually all cellular activities in plants and animals (Goldberg, 1999; Zolnierowicz, 2000; Luan, 2003; DeLong, 2006). There are two kinds of enzymes involved in regulating of phosphorylation that function in opposite ways: protein kinases and protein phosphatases. Protein kinases add phosphoryl groups to amino acids in proteins and serine, threonine and tyrosine are the predominant phosphorylated amino acids in eukaryotic cells. Protein phosphatases remove phosphoryl groups from their substrate proteins. This post-translational modification often changes the activity of the substrate and thus regulates various cell functions, including signal transduction, metabolic pathways and stress responses.

Historically, protein phosphatases have been poorly studied due to the difficulty of detecting phosphate removal *in vitro* and *in vivo*. Since precise regulation of a substrate protein's activity requires both phosphorylation and dephosphorylation, elucidating the function of phosphatases should be equally as important as studying kinases. Recently, the study of phosphatases has gotten more attention. One reason is

because, as a result of sequencing projects for a variety of organisms including mouse, human and *Arabidopsis*, more protein phosphatase genes have been identified and characterized so the knowledge of protein phosphatases is enhanced at the molecular and biochemical level instead of the physiological level only.

Classification of Protein Phosphatases

Protein phosphatases fall into two major groups: tyrosine phosphatases and serine/threonine phosphatases based on the amino acid residues from which the phosphate groups are removed. Animal genomes encode abundant tyrosine phosphatases while the *Arabidopsis* genome encodes only one tyrosine phosphatase but contains at least 80 serine/threonine phosphatases and 18 dual-specificity phosphatases which act on both Tyr and Ser/Thr residues (Kerk et al., 2002; Wang et al., 2003). Serine/threonine phosphatases are further divided into several classes of which PP1, PP2A, PP2B and PP2C (where PP stands for protein phosphatase) are the most well-studied. Serine/threonine phosphatases are classified based on different characteristics (Table 1). Based on substrate preference, serine/threonine phosphatases are divided into two groups. One group contains PP1 and prefers the β-subunit of phosphorylase kinase as substrate. The other group, including PP2A, PP2B and PP2C, prefers the α-subunit of phosphorylase kinase as substrate. Based on ion requirements, Ca^{2+} and Mg^{2+} are required for PP2B and PP2C activity, respectively, while PP1 and PP2A do not have an ion requirement (Table 1). Based on evolutionary relationships from nucleotide or amino acid sequence comparisons, serine/threonine phosphatases are divided into the PPP and

Table 1. Classification of serine/threonine phosphatases (Cohen, 1989; Barford, 1995; Luan, 2003).

Biochemical Classification	Preferred Substrate	Phosphatase Type	Ion Requirement	Inhibitor Sensitivity	Evolutionary Classification
PP1 group	β-subunit of phosphorylase kinase	PP1	None	Peptides 1 & 2; calyculin A; okadaic acid (high concentration)	PPP family
PP2 group	α-subunit of phosphorylase kinase	PP2A	None	Cantharidin; calyculin A; okadaic acid (low concentration)	
		PP2B	Ca^{2+}	EGTA	
		PP2C	Mg^{2+}	EGTA	PPM family

PPM families (Table 1; Cohen, 1989; Barford, 1995; Luan, 2003). Among the protein phosphatase, PP2A and PP1 are the most abundant and are estimated to account for 80-90% of the total protein phosphatase activity in plant cells (MacKintosh and Cohen, 1989). My research focuses on PP2A phosphatases in *Arabidopsis*.

Structure of Protein Phosphatase 2A (PP2A)

PP2A is active as either a heterotrimer consisting of one catalytic (C) subunit and two regulatory (A and B) subunits or as a heterodimer including only the C and A subunits (Janssens and Goris, 2001; Luan, 2003). The C subunit acts as the catalytic subunit which removes phosphate groups from serine or threonine residues on the substrate protein. Based on amino acid sequences, the C subunit is one of the most conserved proteins among eukaryotic organisms, often producing proteins that are 78-98% identical at the amino acid level (Kerk et al., 2002). In *Arabidopsis*, five genes encode the PP2A C subunits (Arino et al., 1993; Casamayor et al., 1994; Perez-Callejon et al., 1998; Kerk et al., 2002), which are named C1, C2, C3, C4 and C5. The five isoforms are divided into two subgroups by amino acid sequence similarity. C1, C2 and C5 belong to one group and are 89% - 93% identical to each other. C3 and C4 form the second group and are 98% identical to each other (Figure 1). As expected, their molecular weights are very close, ranging from 34.9- 35.8 kDa.

The A subunit acts as the scaffold for holoenzyme assembly (Turowski et al., 1997; Price and Mumby, 2000). The crystal structure of the human A subunit showed that it is composed of 15 repeated domains referred to as HEAT repeats. Each domain is about 39 residues long and forms a pair of antiparallel α helices (Groves et al., 1999).

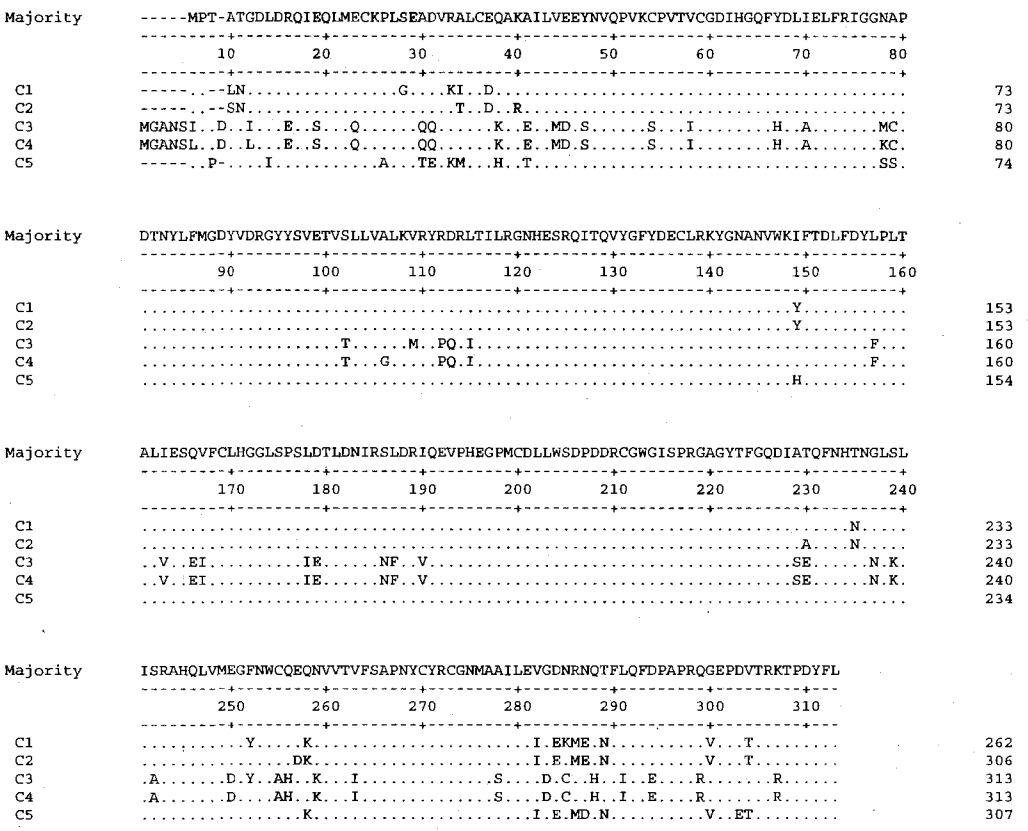

Figure 1. Amino acid alignment of the five *Arabidopsis* PP2A catalytic subunits. Proteins were aligned using Clustal W. Dots represent amino acids identical to the majority. Dashes represent gaps used to maximize the amino acid alignment. C1 = At1g59830, C2 = At1g10430, C3 = At2g42500, C4 = At3g58500, C5 = At1g69960.

Hydrophobic residues in the short turns between each pair of α helices serve as the binding sites for the B and C subunits. B subunits bind to the ten amino terminal repeats and the C subunits interact with the remaining five carboxyl terminal repeats (Groves et al., 1999). In *Arabidopsis*, three genes encode the A subunit and are designated A1, A2 and A3. The molecular weight of the A subunits is approximately 65 kDa and the proteins are 85-93% identical (Figure 2). HEAT motifs were predicted using the online protein motif identification program SMART (Schultz et al., 2000). The A1, A2 and A3 subunits have 12 HEAT repeats.

The B subunit plays a role in regulating the activity of the enzyme as well as in subcellular targeting and substrate specificity (Janssens and Goris, 2001). The crystal structure of a human B subunit (B′/B56/PR61-γ 1) shows an elongated, superhelical structure comprised of 18 α helices (Xu et al., 2006). Surprisingly, the structure also contains eight HEAT-like motifs closely resembling those of the human Aα subunit (Xu et al., 2006). In *Arabidopsis*, seventeen genes encode B regulatory subunits. Based on amino acid sequence, the B subunits are divided into three subgroups which have little inter-group sequence similarity and are named alphabetically within each subgroup using Greek letters (Terol et al., 2002). The first subgroup, B, includes two members. They are approximately 56 kDa and are 80% identical at the amino acid level (Figure 3; Rundle et al., 1995; Corum et al., 1996). The second subgroup, B′, includes nine proteins (Haynes et al., 1999; Terol et al., 2002). Their molecular weights are approximately 58 kDa. The nine proteins have a wider range (48-86%) of amino acid identity than the B subunits, primarily because of their divergent N- and C-termini (Figure 4). The third subgroup, B″,

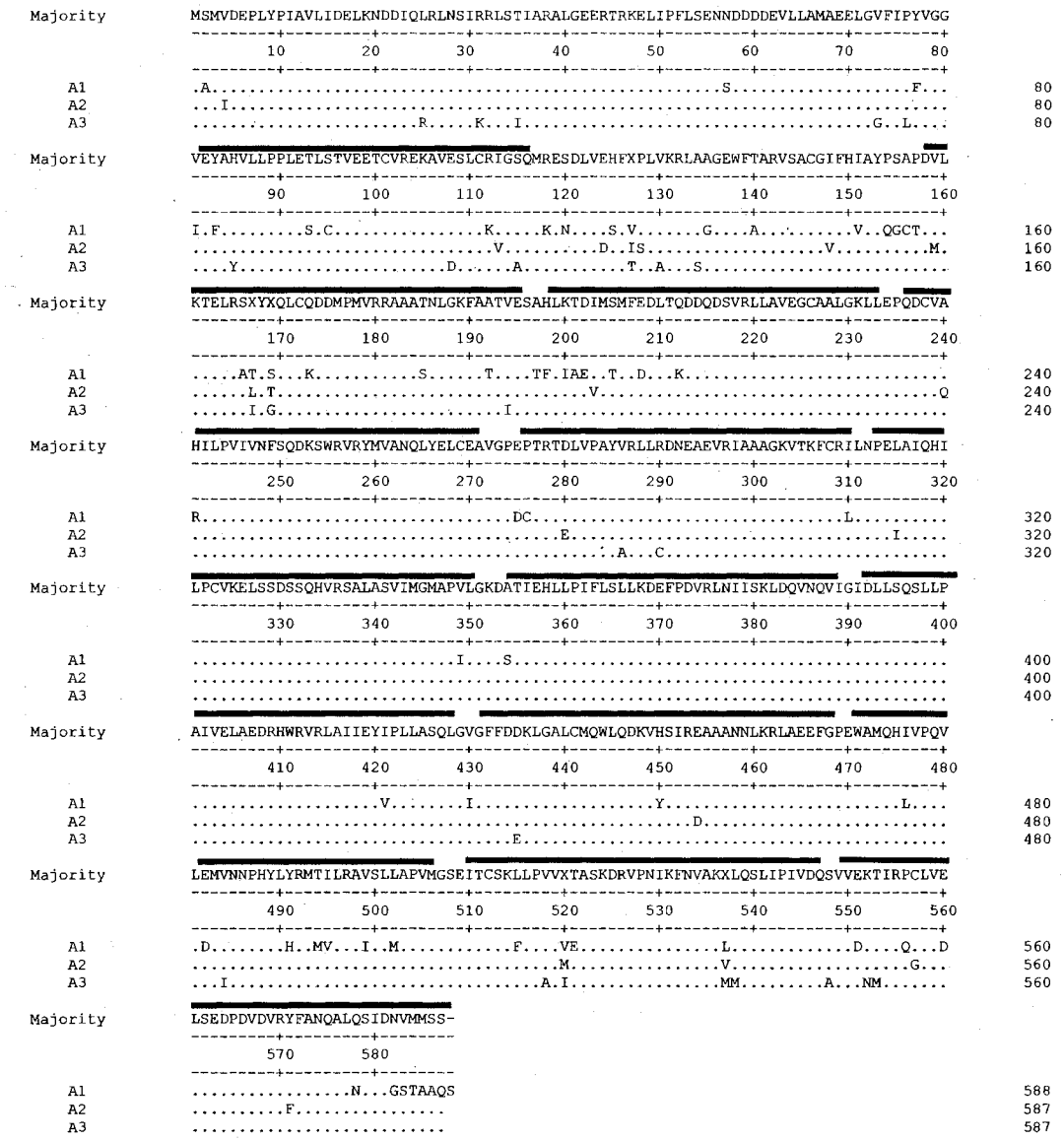

Figure 2. Amino acid alignment of the three *Arabidopsis* PP2A A subunits. Proteins were aligned using Clustal W. Dots represent amino acids identical to the majority. Black bars indicate HEAT motifs. A1 = At1g25490, A2 = At3g25800, A3 = At1g13320.

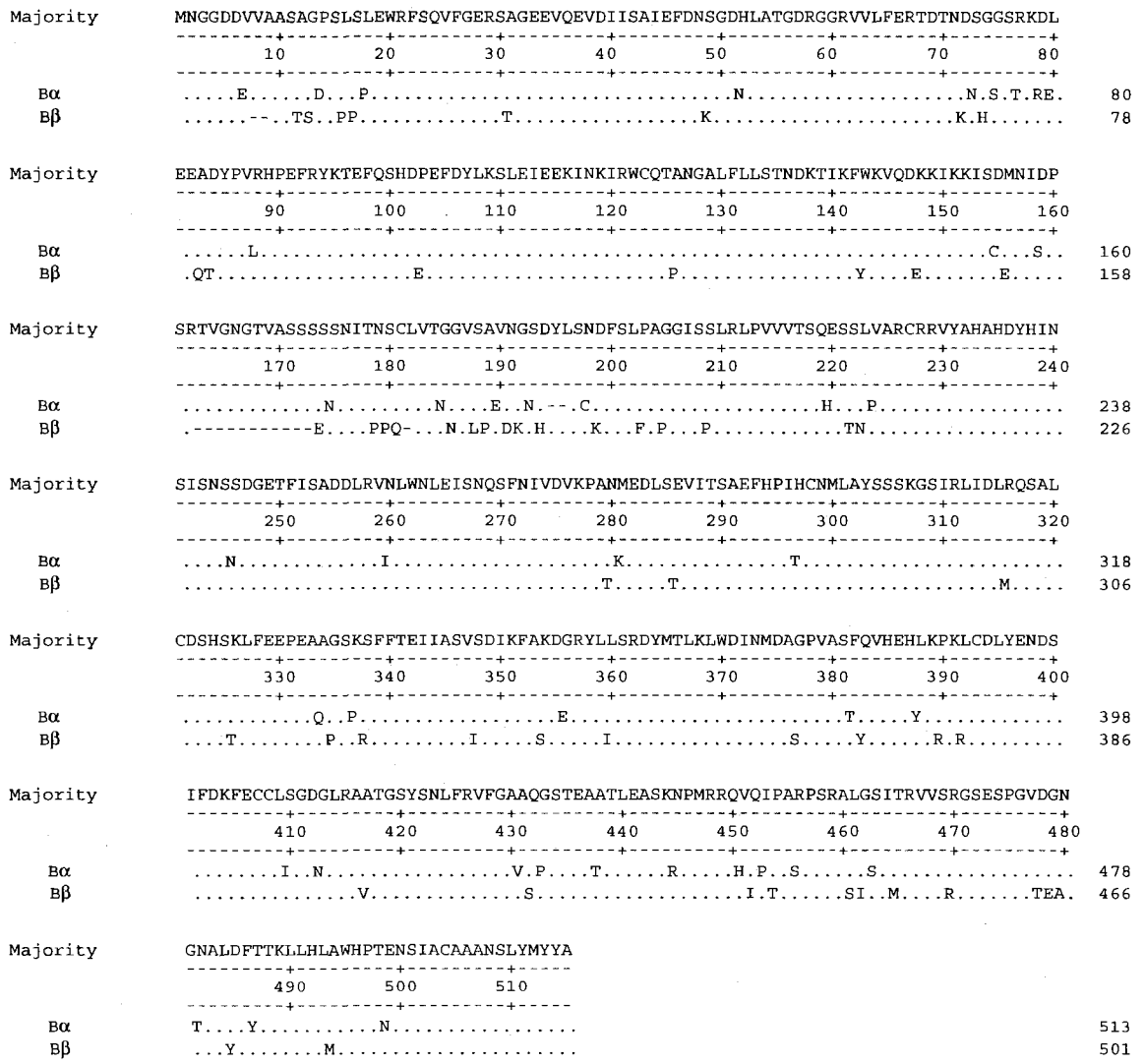

Figure 3. Amino acid alignment of the two *Arabidopsis* PP2A B subunits. Proteins were aligned using Jotun Hein. Dots represent amino acids identical to the majority. Dashes represent gaps used to maximize the amino acid alignment. Bα=At1g51690, Bβ=At1g17720.

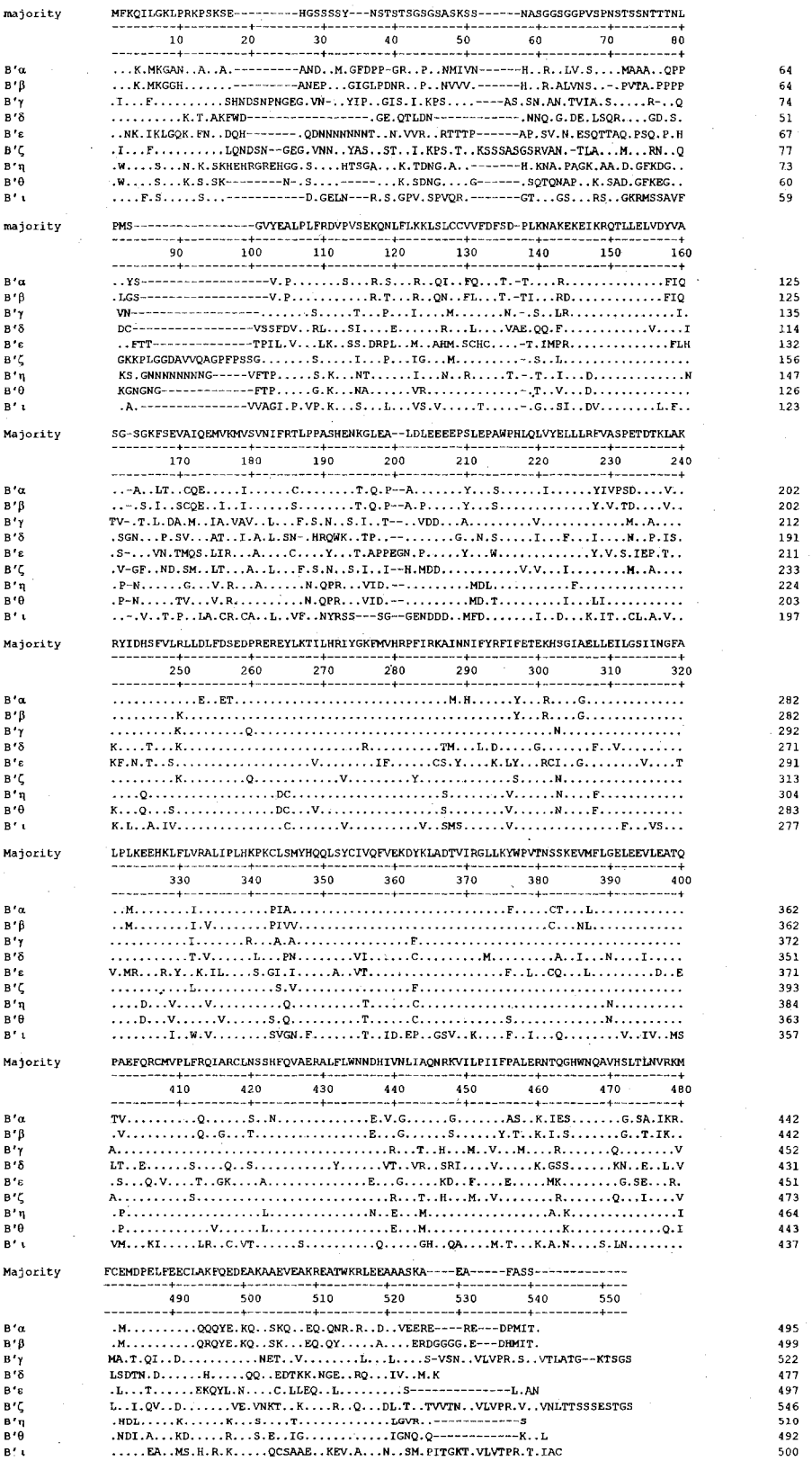

Figure 4. Amino acid alignment of the nine *Arabidopsis* PP2A B′ subunits. Proteins were aligned using Clustal W. Dots represent amino acids identical to the majority. Dashes represent gaps used to maximize the amino acid alignment. B′α=At5g03470, B′β=At3g09880, B′γ=At4g15415, B′δ=At3g26030, B′ε=At3g54930, B′ζ=At3g21650, B′η=At3g26020, B′θ=At1g13460, B′ι=At5g25510.

contains five genes. Their molecular weights are about 62 kDa and they range from 66-99% identity (Figure 5). There is a potential sixth member in B″ subgroup named B″ζ. B″ζ encodes a protein named TONNEAU or TON2 (Camilleri et al., 2002) and is reported to be a member of the PP2A B″ subgroup although amino acid sequence comparison shows a very low level (20%) of amino acid sequence identity with B″ subunits from plants and animals and no similarity to B or B′ subunits. There is evidence from the yeast two-hybrid system that the TON2 protein can interact with the A1 subunit (Camilleri et al., 2002), but it is still unclear whether TON2 is a PP2A B″ subunit or simply a PP2A A subunit interactor. So for this reason, I have not included B″ζ in the B″ subgroup alignment.

Considering the number of genes that have been identified in *Arabidopsis* for each of the three subunits, 255 heterotrimers theoretically could be formed. Little is known about which heterotrimers (or heterodimers) actually are formed *in vivo*. Using the yeast two-hybrid system to detect protein-protein interactions, the B′β, B′γ, C1 and C5 subunits were all shown individually to interact with the A1 subunit (Haynes et al., 1999), but no further studies have extended this work and no heterotrimers have been identified *in vivo*.

Regulation of PP2A

Protein phosphatase 2A is a highly-regulated enzyme and this regulation could occur at different levels. First, there is evidence that all three groups of PP2A subunits show spatially and temporally distinct expression patterns. For example, expression of the C1 and C3 subunit genes is higher than that of the C2 subunit in *Arabidopsis*

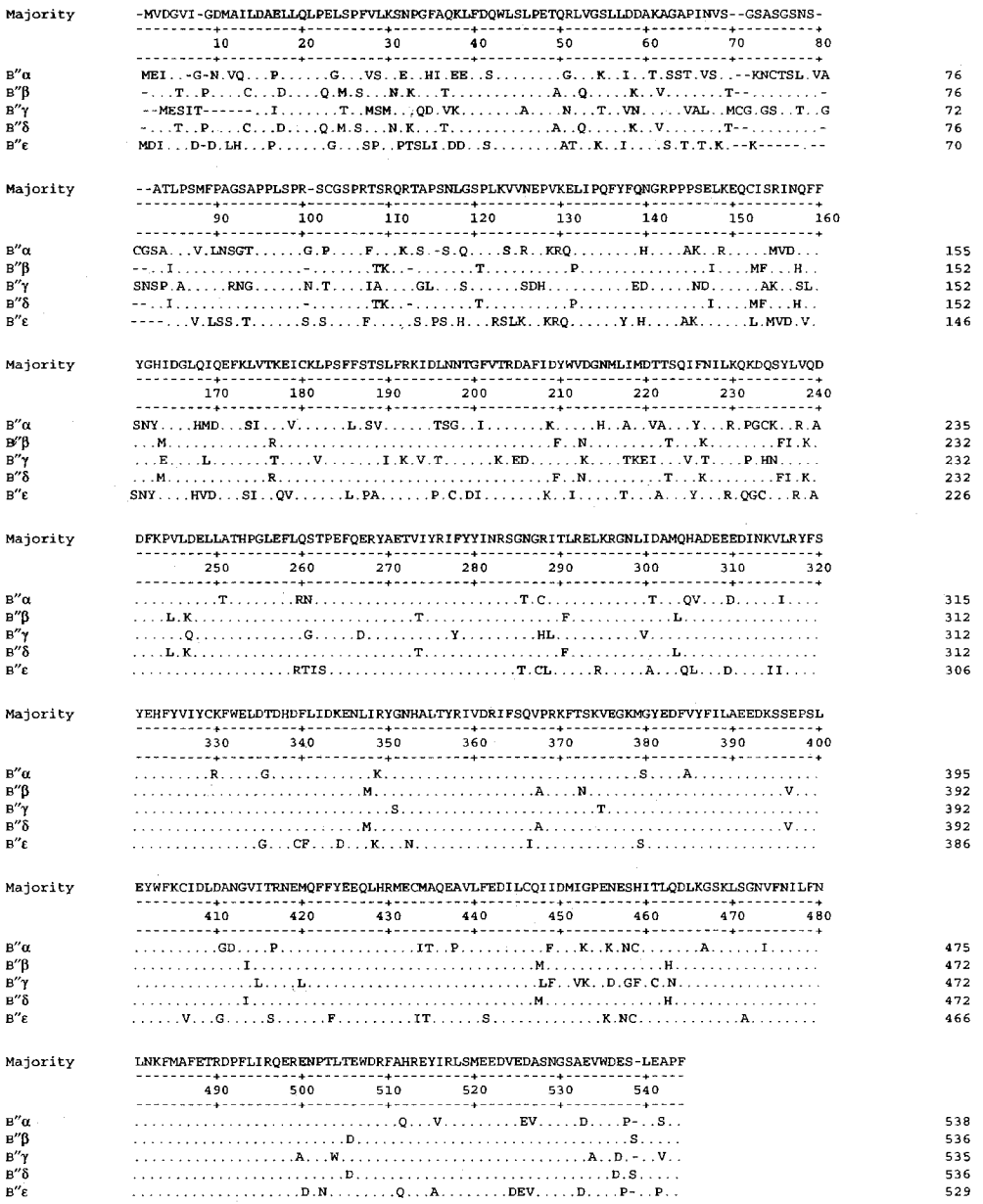

Figure 5. Amino acid alignment of the five *Arabidopsis* PP2A B″ subunits. Proteins were aligned using Clustal W. Dots represent amino acids identical to the majority. Dashes represent gaps used to maximize the amino acid alignment. B″α=At5g44090, B″β=At5g28900, B″γ=At1g54450, B″δ=At5g28850', B″ ε=At1g03960.

seedlings by northern blot (Arino et al., 1993). Northern blots also showed that the B′ genes are expressed differently in many tissues. For example, B′α is expressed in the roots, stem, and leaves with highest expression detected in the leaves, while B′β mRNA is at higher levels in the cotyledons and flowers than in the roots, stems, and leaves (Latorre et al., 1997).

Second, regulation of PP2A activity can occur at the level of enzyme subunit composition. As mentioned before, PP2A can function as either a heterodimer or heterotrimer. The B subunit plays a role in regulating the activity of the enzyme as well as in subcellular targeting and substrate specificity. Unlike the A and C subunits, amino acid sequence similarity between different subfamilies of the B regulatory subunits is low, and the expression levels of various B subunits are highly diverse depending upon cell types and tissues (Janssens and Goris, 2001). Thus, the diversity of B subunits is a major determinant of enzyme diversity through its impact on PP2A subunit composition. The crystal structure of the B subunit also provides evidence that this subunit determines the holoenzyme's specificity (Cho and Xu, 2007). The crystal structure of the human PP2A heterotrimeric holoenzyme shows that the B and C subunits form a structure in which the HEAT repeats of the B subunit are brought into close proximity with the C subunit active site and provide the docking site for PP2A substrates. Substrate specificity is determined through binding of specific substrates to different areas on the surface of the B-C complex (Cho and Xu, 2007).

Third, PP2A subunit composition is regulated by post-translational modifications including methylation and phosphorylation of the C subunits. The C-terminus of the

PP2A C subunit can be both methylated and phosphorylated. The type of modification determines which specific B subunit binds to the A-C heterodimers with the result of producing holoenzymes with specific catalytic properties, subcellular locations, and substrate specificities (Ogris et al., 1997; Chung et al., 1999).

Function of PP2A in Fungi and Animals

Using the PP2A inhibitor okadaic acid or using genetic approaches, several roles have been described for PP2A proteins in animals and fungi. Here I present a few examples of the roles identified for PP2A in animal and yeast cells.

First, PP2A is required for pre-mRNA splicing. Pre-mRNA splicing is an essential step for expression of the vast majority of genes. This process involves the spliceosome, a macromolecular complex containing five snRNAs and several hundred proteins (Zhou et al., 2002; Jurica and Moore, 2003). The spliceosome and pre-mRNA assemble together to conduct a two-step intron splicing process which yields ligated exons and a lariat intron. PP2A is required mainly for the second step of splicing and several proteins such as the U2 and U5 snRNPs and SAP155 in the spliceosome are substrates of PP2A (Shi et al., 2006).

Second, PP2A functions in the regulation of the cell cycle. For example, the G_2/M transition of the cell cycle is governed by M-phase-promoting factor (MPF) which is a complex including $p34^{cdc2}$ kinase and cyclin B. PP2A functions as a negative regulator to keep M-phase-promoting factor (MPF) in its inactive state (Millward et al., 1999; Janssens and Goris, 2001; Garcia et al., 2003; Janssens et al., 2005). Through mass

spectroscopy-based proteomic analysis, PP2A was identified to be present in human interphase centrosomes, functioning in cell division and cell-cycle progression (Rieder et al., 2001, Andersen et al., 2003). PP2A is also required for bipolar spindle assembly and the maintenance of short microtubules during metaphase in *Xenopus* egg extracts (Tournebize et al., 1997).

Third, PP2A is a tumor suppressor and PP2A mutants are associated with cancers in humans. For example, mutations in either of the two human A regulatory subunits can result in compromised binding of either the regulatory or catalytic subunits and have been found in 15% of primary lung tumors, 6% of lung tumor-derived cell lines, and 15% of colorectal carcinomas (Wang et al., 1998; Calin et al., 2000; Takagi et al., 2000; Colella et al., 2001; Ruediger et al., 2001; Suzuki and Takahashi, 2003). In addition, an N-terminally truncated human B′ subunit promotes genetic instability and causes tumor progression (Ito et al., 2003; Chen et al., 2004; Koma et al., 2004).

Functions of PP2A in Plants

The functions of PP2A in plants are still not well understood. One common experimental approach has been to use the inhibitor okadaic acid (OA) followed by comparison of phenotypes to an untreated control. One potential complication with this approach is that OA also inhibits PP1, another kind of abundant protein phosphatase (Table 1). However, the sensitivity of PP2A to OA (~1-3 nM) is approximately 100-fold greater than that of PP1 (~60-500 nM). The protein phosphatase PP4 is also inhibited by OA at low concentration (~0.2 nM; Brewis et al., 1993) but since PP4 is much less abundant than PP2A in plants, it is usually assumed that effects observed at low OA

concentrations are due primarily to inhibition of PP2A. Based on OA inhibitor experiments, PP2A has been shown to be involved in a wide range of functions in plants including cytoskeletal organization (Smith et al., 1994; Foissner et al., 2002), regulation of carbon and nitrogen metabolism (Siegl et al., 1990; Huber et al., 1992), production of plant defense compounds triggered by fungal elicitors (MacKintosh et al., 1994), ion channel activity (Li et al., 1994), and cold responses (Monroy and Dhindsa, 1995). However, the specific PP2A heterodimers or heterotrimers involved in each of these processes have not yet been defined.

Using a forward genetic approach, which starts with a mutant phenotype and works toward identifying the mutated gene, a PP2A A subunit mutant phenotype was first reported in 1996. This PP2A *A1* subunit mutant was named *rcn1* because it was identified due to its root curling ability in the presence of naphthylphthalamic acid (NPA), an auxin transport inhibitor. In the absence of NPA, roots of wild type seedlings grow to the bottom of the Petri dish and then grow in a curling pattern on the bottom of the plate. In the presence of NPA, roots of wild type seedlings fail to curl and grow in a straight line along the bottom of the plate, while roots of the *a1* mutant (*rcn1*) exhibit a tight curling pattern. The *a1* mutant was subsequently shown to affect multiple auxin-regulated processes including root and hypocotyl elongation, apical hypocotyl hook formation in the dark, gravity responses, and lateral root growth (Garbers et al., 1996; Deruere et al., 1999; Rashotte et al., 2001). Increased auxin transport was detected in etiolated *a1* mutant hypocotyls (Muday et al., 2006). The *a1* mutant has reduced phosphatase activity compared to wild type plants indicating that the phenotype is likely due to an enzyme

deficiency (Deruere et al., 1999). The phenotypes of T-DNA insertion mutants in the other two A subunit genes, *A2* and *A3*, are largely normal and do not resemble that of the *A1* mutant (Zhou et al., 2004). However, *a1a2* and *a1a3* double mutants exhibit extreme abnormalities in all stages of development while the *a2a3* double mutant exhibits only slight defects. These results indicate that, although the A1, A2 and A3 subunits are expressed in all *Arabidopsis* organs, the A1 subunit makes the greatest contribution to PP2A function while the contributions of the A2 and A3 subunits are only apparent in the absence of A1 (Zhou et al., 2004).

Besides auxin responses, several reports indicated that the A1 subunit may be involved in responses to two other plant hormones: ethylene and abscisic acid (ABA; Kwak et al., 2002; Larsen and Cancel, 2003). In ethylene signaling, the A1 subunit functions as a negative regulator and the mutant displays increased sensitivity and amplitude of response to ethylene (Larsen and Cancel, 2003). In ABA signaling, the *a1* mutant is impaired in ABA-induced stomatal closing and is less sensitive to ABA inhibition of seed germination. Furthermore, other ABA responses like cytosolic Ca^{2+} influx, expression of ABA-responsive genes, and ion channel activation are reduced in the *a1* mutant (Kwak et al., 2002). These results show that the A1 subunit functions as a positive regulator in several ABA responses.

Besides A subunit mutants, some phenotypes were identified from B and C subunit mutants. B''ζ encodes a protein named TONNEAU or TON2 (Camilleri et al., 2002) and is reported to be a member of the PP2A B'' subgroup. The *ton2* mutant exhibits disruption of microtubules in the cortical cytoskeleton, leading to dwarf plants

with abnormally shaped cells (Camilleri et al., 2002). As mentioned before, it is not known whether TON2 is a PP2A B″ subunit or simply a PP2A interacting protein. So for this reason, I have not included TON2 in the B″ subgroup.

Recently, a catalytic C2 subunit has been identified as a negative regulator of the ABA pathway (Pernas et al., 2007). A recessive mutant was identified with a T-DNA-mediated disruption of the PP2A C2 gene. The C2 transcript was absent in the *pp2ac-2* mutant and led to a significant decrease of PP2A activity, about 20% lower than in wild-type plants. The *pp2ac-2* mutant is hypersensitive to ABA in multiple ABA-regulated processes such as lateral and primary root growth, seed germination, and responses to drought and high salt and sugar stresses. More evidence comes from C2 overexpressing plants. Compared with wild type lines, total PP2A activity was increased by 40-50% in the overexpressing lines (Pernas et al., 2007). As expected, transgenic plants overexpressing the C2 protein are less sensitive to ABA than wild type, a phenotype that is manifested in all of the above-mentioned physiological processes. With a DNA microarray hybridization approach, C2 was revealed to negatively regulate ABA-dependent gene expression (Pernas et al., 2007). The evidence from phenotypic, genetic and gene expression data strongly suggests that PP2A C2 is a negative regulator of the ABA signaling pathway.

Rationale and Significance

In plants, protein phosphorylation is involved in almost all signaling pathways. Phosphorylation and dephosphorylation of proteins often serve as "on-and-off" switches to regulate cellular activities. These two reversible processes must strike a balance in

cells and if this balance is altered, cellular processes can be adversely affected. Despite the remarkable importance and significance of dephosphorylation, much less research has been done on phosphatases compared to kinases. My research focuses on protein phosphatase 2A (PP2A) which is one of the most abundant types of serine/threonine phosphatases in all eukaryotic cells, comprising 1% of the total protein content in mammalian cells (DePaoli-Roach et al., 1994; Lin et al., 1998). The importance of PP2A has been intensively studied in animal and fungal cells but is poorly recognized in plant cells. Our knowledge of PP2A function in plants is growing but is still far from complete. Data from gene expression using RT-PCR and northern blotting has provided some information about the tissues and development stages in which PP2A genes are expressed (Arino et al., 1993; Casamayor et al., 1994; Smith and Walker, 1996; Latorre et al., 1997; Toth et al., 2000). Additional experiments using a PP2A inhibitor okadaic acid have provided evidence that PP2A is involved in other plant processes including cytoskeletal organization, cold responses, etc. All of these results point to potential functions of PP2A in plants; however the specific PP2A genes or heterotrimers involved in these processes are not known since the PP2A inhibitor okadaic acid is not specific and inhibits all PP2As. A genetic approach has been used to identify PP2A mutants in *Arabidopsis*. So far, only a few mutants with phenotypes have been identified to date. These mutants showed that heterodimers or heterotrimers containing specific PP2A subunits are involved in responses to the plant hormones auxin, ABA, ethylene and in the control of cytoskeletal structure. It is likely that more mutants of PP2A will be identified which will provide additional evidence of PP2A function in plants.

The main goal of my Ph.D. research was to determine the roles of PP2A C subunits in plants. I used the *Arabidopsis* T-DNA mutant collections to identify plant lines with T-DNA insertions in C subunit genes. Homozygous mutant plants were then analyzed by RT-PCR to detect the presence of transcripts. Knockout mutant plants were screened using various hormone and chemical treatments as well as under different growth conditions and their phenotypes compared with wild type plants. Once a phenotype was identified, complementation tests were conducted to confirm that the mutant phenotype was due to loss of PP2A C function and the phenotype was further characterized. These studies will contribute to our overall understanding of phosphatase function in plants.

CHAPTER II

IDENTIFICATION OF PROTEIN PHOSPHATASE 2A C SUBUNIT MUTANTS IN *ARABIDOPSIS THALIANA*

Introduction

Forward genetics begins with a mutant phenotype and seeks to identify the gene which causes the altered phenotype. This approach has been intensively used for over a century and has achieved many exciting results in all areas, including plant biology. With the completion of the *Arabidopsis* genome sequencing project (The *Arabidopsis* Genome Initiative, 2000), the opportunity to conduct research with a reverse genetics approach became available. In contrast to forward genetics, reverse genetics begins with a mutated gene (gene knockout) and seeks to identify any associated phenotypes. In this study, a gene knockout approach was used to identify the phenotype of a PP2A C subunit gene mutant in plants.

Insertional mutagenesis uses transferred DNA or T-DNA to create mutants. *Agrobacterium tumefaciens* contains a resident Ti plasmid and, as part of its life cycle as a plant pathogen, transmits T-DNA, a segment of the Ti plasmid, into plant cells where it becomes integrated into the plant cell genome (Azpiroz-Leehan and Feldmann, 1997). T-DNA is usually greater than 5 Kb in size. When T-DNA is transferred into the plant genome by *Agrobacterium*, it can disrupt gene function depending on the insertion site.

The presence of this large foreign DNA fragment in a genome might result in no transcript, a mis-spliced transcript or a transcript that is improperly terminated. In general, T-DNA is likely to disrupt mRNA production when inserted in introns or exons. T-DNA can be used as a mutagen by modifying its structure to contain new genes, one of which is usually a selectable marker gene which can be used to identify plants with T-DNA inserts.

Using T-DNA as a mutagen has several advantages. First, the junction between the T-DNA and its insertion site in the genome can be determined by sequencing and since the *Arabidopsis* genome is fully sequenced, the insertion site of T-DNA can be easily identified (Krysan et al., 1999). Second, since T-DNA insertions are stable and heritable, the mutation within the gene of interest can be maintained over generations (Krysan et al., 1999). Third, transformation of *Arabidopsis* is easy and a large population of T-DNA transformed lines can be generated. Thus, there is a reasonable chance that a plant carrying a T-DNA insert within any gene of interest will be found depending on the size of the mutagenized population (Krysan et al., 1999).

The T-DNA insertion collection (Alonso et al., 2003) created at the Salk Institute is one of the available T-DNA collections and contains more than 88,000 T-DNA insertions in the *Arabidopsis* genome. T-DNA insertion sites were determined using TAIL-PCR. The Salk Institute Genome Analysis Laboratory (SIGnAL) online database contains the predicted sites of insertion of these T-DNAs in the *Arabidopsis* genome. The data are available via a web-accessible graphical interface called T-DNAExpress (http://signal.salk.edu/cgi-bin/tdnaexpress) that provides both text and DNA searches of

the insertion sequence database. Seeds of the T-DNA insertion lines are deposited in the *Arabidopsis* Biological Resource Center (ABRC) at Ohio State University and can be requested by researchers. In this study, PP2A C subunit mutant lines were ordered from ABRC after identifying their T-DNA insertion sites from the SIGnAL online database. Most of the PP2A mutant lines I have been working on were generated by the Salk Institute and seeds were obtained from ABRC. One mutant line was ordered from GABI, a different T-DNA mutant collection. Similar to the SIGnAL website, the T-DNA Insertion Sequence Database created by Bielefeld University in Germany catalogs T-DNA insertions in the *Arabidopsis* genome. The sites of T-DNA insertion are identified at http://www.gabi-kat.de/simplesearch/simplesearch.html. Seeds of T-DNA insertion lines are deposited in the Nottingham *Arabidopsis* Stock Center (NASC) at the University of Nottingham, UK and can be distributed to researchers.

The mutant lines available from seed stock centers usually are segregating T_3 lines with a mix of hemizygous and homozygous seeds. Since most mutations are expected to be recessive, obtaining a homozygous mutant plant is essential for further experiments. The first step is to determine if the plant is homozygous or hemizygous for the T-DNA insertion by PCR by looking for the presence of a wild type copy of the gene. A pair of gene-specific primers, with one primer located upstream and the other downstream of the predicted T-DNA insertion, is designed. The distance between these primers should be less than 1 Kb for ease of amplification. A PCR product between the two primers should be amplified in hemizygous plants since hemizygous plants retain one wild type copy of the gene. For plants homozygous for the T-DNA insertion, no PCR product (i.e., a negative result) is expected due to the inability of *Taq* DNA polymerase to

amplify across the large T-DNA insertion (~5 kb). In this study, I identified homozygous PP2A mutants for all five C subunit genes.

Materials and Methods

Arabidopsis Strains and Growth

All *Arabidopsis* strains used in this study, including wild type and mutant lines were of ecotype Columbia. *Arabidopsis* seeds were grown on Petri plates containing Low Sucrose MS medium [0.44% (w/v) Murashige & Skoog basal salt mixture with vitamins (Caisson Laboratories, Inc., North Logan, UT, Cat. No. MSP0506), 0.1% (w/v) sucrose and 0.8% (w/v) washed agar, pH 5.7]. After autoclaving, kanamycin was added to a final concentration of 50 μg/ml before pouring plates. Generally, 25 ml of medium was poured into a 100 x 25 mm Petri plate and 10-15 sterile seeds were placed on each plate.

Seeds were sterilized in a 1.5 ml microcentrifuge tube. First, seeds were mixed with 70% (v/v) ethanol containing one drop of 10% (v/v) Triton X-100 for 5 min. After removing the 70% ethanol, the seeds were mixed with 100% ethanol for 5 min. The seeds were spread on a piece of sterile filter paper and dried before being sprinkled on the plates. Seeds on plates were stratified at 4°C in darkness for three days. After cold treatment, the plates were placed in a growth room at 25°C ±2°C, 12 hour photoperiod, and fluorescent light at 80±10 μmol m^{-2} s^{-1}. After 10 days, the seedlings were transplanted into artificial growth medium [perlite (Whittmore Company, Inc, Lawrence, MA) and Metromix 360, 1:1] and grown at 21°C, 16 hour photoperiod, and 100±10 μmol m^{-2} s^{-1} of fluorescent light. The plants were watered as needed with commercial plant food.

Rapid DNA Extraction

Arabidopsis DNA was extracted using a rapid DNA preparation method (Klimyuk et al., 1993). A piece of *Arabidopsis* leaf approximately 0.5 cm^2 was snipped off using the top of a 1.5 ml microfuge tube. Forty µl of 0.25 M NaOH was added and the leaf piece was smashed with a pipette tip until the liquid looked slightly green. The sample was boiled for 30 seconds and subsequently neutralized by addition of 40 µl 0.25 M HCl and 20 µl 0.5 M Tris-HCl (pH 8.0) containing 0.25% (v/v) Nonidet P-40. The sample was boiled an additional 2 minutes and stored at 4°C. The sample was incubated at 100°C for 2 minutes prior to use as PCR template. This method was used for short-term storage of DNA (usually several weeks).

Primer Design for Knockout Mutant Identification

Two PCR primers specific to the left border of the T-DNA, JMLB1and JMLB2, were designed by the Salk Institute Genome Analysis Laboratory (SIGnAL). Primers specific to PP2A mutant lines were designed using the program Primer Design (Lasergene, Madison, WI). All primers were synthesized by Integrated DNA Technologies (IDT, Coralville, IA).

PCR Amplification

PCR amplification was performed in a PTC-100 programmable thermal controller (MJResearch, Watertown, MA). Each reaction contained of 6.3 µl of water, 1 µl of 10x home-made Taq buffer (500 mM KCl, 100 mM Tris-HCl, pH 8.5, 15 mM $MgCl_2$), 1.2 µl

of 2.5 mM dNTP mix (Roche, Indianapolis, IN), 0.25 µl of both the forward and reverse primers at 12 pmol/µl, and 1 µl of template DNA from the rapid DNA isolation for a total volume of 10 µl. A hot start cocktail was added to each reaction after the temperature of the thermal controller block reached 95°C. Each aliquot of the hot start cocktail included 4 µl of water, 0.5 µl of 10x home-made Taq buffer and 0.5 µl of home-made Taq. Primers used in these reactions are listed in Table 2. PCR was performed according to the following program: hot start at 94°C for 5 minutes, followed by 36 cycles at 94°C for 30 seconds, 50°C -75°C for 30 seconds depending on the primers' annealing temperatures (Table 2) and 72°C for 2 minutes. The program finished with 72°C for 5 minutes.

PCR products were separated by size on 1% agarose gels in 1X TAE buffer (40 mM Tris-acetate (pH 8.0), 1 mM EDTA) at 8 V/cm. Five µl of each reaction and 1 µl of loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, 15% Ficoll) was loaded into each well. A 1 Kb ladder (Invitrogen, San Diego, CA) was used as a size marker. After separation, the DNA was visualized by staining the gel with ethidium bromide followed by exposure to UV light.

Sequencing Reactions

DNA sequencing reactions were performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (ABI, Foster City, CA). Each reaction contained 1 µl of sequencing reagent premix, 3 µl of 5x buffer, 5-20 ng of template DNA and 2 µM of primer, for a total volume of 10 µl. The PTC-100 thermal controller was programmed for an initial step of 95°C for 1 minute, followed by 25 cycles at a denaturation temperature

Table 2. Primers used for identification of PP2A homozygous mutants.

Primer Name	Primer Sequence	Annealing Temperature (°C)
JMLB1	GGCAATCAGCTGTTGCCCGTCTCACT	82
JMLB2	TTGGGTGATGGTTCACGTAGTGGGCCATCG	94
T-DNA GABI800G05	CCCATTTGGACGTGAATGTAGACAC	74
2AC11	TTTCACTTCCGCACTTTTCTTCA	64
2AC12	ATGGGTTTTTCGTCTTTGGTATCA	64
2AC21	TAACCGGGTCATAAAACATT	54
2AC22	TACGGCGGATAAAGAAGAT	54
2AC31	TTATGAAGCGAAGGCAAAAGTCT	64
2AC32	TACCAGGCACATAAAAACACGAG	66
2AC35	GTATCAAATCTCGTCGCCATCACA	70
2AC36	CAAAGCCAATTCTCCAACCCATAG	70
2AC43	CTACAGAGAAATGCGGACGAT	62
2AC44	TTGGGCAAAGACCTGAATGAA	60
2AC47	AACGAGGAGGATATAATGGTCAAA	66
2AC48	TCCGGAAAACAGATCAGAAACGAG	70
2AC49	CGGACACAACGCGATAAAATAGAC	70
2AC410	AAGAAAACGAAAAGGTGCGAGGAA	68
2AC411	CCACTCGCCCCTTTACCTCAC	68
2AC412	TCATCTTTCCCCTCTTTTACTTCC	68
2AC511	CGCATGTACGATAAGGTTCAGTGG	60
2AC512	TCTCGGATCCGGTTTGTGTTTG	66
2AC513	TCGGTGGTTCTTCTCCTGATACT	68
2AC514	TGGCACCAATTAAAACCTTCC	58

of 95°C for 10 seconds, an annealing temperature of 50°C for 5 seconds, and an extension temperature of 60°C for 4 minutes. After the reaction was complete, the products were ethanol precipitated by the addition of 1 μl of 125 mM EDTA (pH 8.0), 1 μl of cold 3 M sodium acetate (pH 5.2) and 25 μl of 100% ethanol and then incubated at room temperature for 15 minutes. The reaction was centrifuged for 30 minutes at 3,000xg and the pellet was washed with 70 μl of 70% ethanol, centrifuged for 15 minutes, vacuum dried and dissolved in 10 μl of deionized formamide. Sequencing was performed by the UNH DNA Sequencing Facility.

Results

Identification of Homozygous Mutant PP2A C Subunit Plants

Seeds of the PP2A C subunit mutant lines (Table 3) were ordered from ABRC (http://www.biosci.ohio-state.edu/pcmb/Facilities/abrc/abrchome.htm) or from GABI (http://www.GABI-Kat.de). The predicted T-DNA insertion sites in the PP2A C subunit genes were identified from the T-DNA Insertion Sequence Database. The mutant lines available from ABRC are usually segregating T_3 lines with a mixture of hemizygous and homozygous seeds. The mutant lines available from GABI are usually homozygous seeds. It is necessary to use homozygous mutant plants for further phenotypic screening since most mutations are expected to be recessive.

After obtaining seeds from ABRC, ten seeds of each genotype were germinated on a Low Sucrose MS plate each genotype of containing 50 μg/ml kanamycin. If no seeds germinated due to silencing of the kanamycin-resistance gene (see Discussion for details), another ten seeds were germinated on a plate containing only Low Sucrose MS. Seeds from GABI were germinated on a Low Sucrose MS plate because no appropriate antibiotic were available in my lab. After ten days, healthy seedlings were transplanted to artificial growth medium. When the leaves were large enough, a leaf sample was removed and DNA was isolated with the rapid DNA isolation method. PCR was used to determine if the plant was homozygous or hemizygous for the T-DNA insertion by first looking for the presence of a wild type copy of the gene. Gene-specific primers flanking

Table 3. PP2A C subunit mutant lines, allele numbers, primers and PCR products.

Subunit	Gene Identifier	T-DNA Identifier Number	Allele Number	Forward Primer and Reverse Primer	PCR Product Size (bp)
C1	At1g59830	Salk-102599	*c1-1*	2AC11 and 2AC12	908
C2	At1g10430	Salk-150673	*c2-1*	2AC21 and 2AC22	221
C3	At2g42500	Salk-069250	*c3-1*	2AC31 and 2AC32	875
		Salk-127619	*c3-2*	2AC35 and 2AC36	855
		Salk-135719	*c3-3*	2AC35 and 2AC36	855
C4	At3g58500	Salk-035009	*c4-1*	2AC43 and 2AC44	715
		GABI-800G05	*c4-2*	2AC411 and 2AC42	995
		Salk-018364	*c4-3*	2AC49 and 2AC410	1274
		Salk-130933	*c4-4*	2AC47 and 2AC48	228
		Salk-138586	*c4-5*	2AC49 and 2AC410	1274
		Salk-145903	*c4-6*	2AC47 and 2AC48	228
C5	At1g69960	Salk-013178	*c5-1*	2AC511 and 2AC512	449
		Salk-139822	*c5-2*	2AC513 and 2AC514	1240

the T-DNA insertion were designed. For hemizygous plants, a product was amplified since the plant retained one wild type copy of the gene. For homozygous plants, no PCR product (i.e., a negative result) was expected due to the inability of *Taq* DNA polymerase to amplify across the large T-DNA insertion (~5 kb). This negative result was inconclusive since a failed reaction could also be responsible for no PCR product. Therefore, several control reactions were performed simultaneously. The first control tested the amplification ability of the primers using wild type DNA as template. The second control tested the ability of the DNA to function as a template in PCR using primers for any other gene. The genomic sequences of the five C subunit genes with the T-DNA insertion sites and primers marked are in Figures 6-10.

An example of the genotyping of the PP2A mutant allele *c4-1* is shown in Figure 11. Seeds from a plant containing a T-DNA insertion in the *C4* gene were grown on a plate containing kanamycin. About one fourth of the plants were killed, likely because they were segregating wild type plants with no T-DNA insertions. The surviving seedlings were transplanted to artificial growth medium and DNA was isolated from leaf tissue. PCR was performed using two PP2A C4-specific primers, 2AC43 and 2AC44 (Figure 9), to detect the presence of a wild type copy of the *C4* gene. No wild type copy of the *C4* gene was amplified in two of the eight plants (Figure 11A, lanes 3 and 4) although a product was amplified in the other six plants with the expected product size of 715 Kb. This indicates that these two plants are potentially homozygous for the T-DNA insertion. However, the lack of a wild type PCR product in Figure 11A, lanes 3 and 4 could also be due to poor quality of either the PCR or the template DNA, so control PCRs were set up. The first positive control used the PP2A C4 primers on wild type DNA and

taaatatccattttgtgtgatatgagagaaacaaaaccaaagacgagccagagccacctctaacaccatcggcgacttctcct
cctactcctccgtcgccgacgtgttcgatttacaacgattcggtggctattggttgtaagatttagaattaaaggttaaaggagac
*gacgacgagag***ATG**CCGTTAAACGGAGATCTCGACCGTCAGATCGAACAGCTAAT
GGAGTGTAAGCCGTTAGGTGAAGCAGACGTGAAGATCCTTTGCGATCAAGCT
AAAGCGATTCTTGTTGAGGAATATAATGTTCAACCGGTTAAGTGTCCGGTTAC
GGTATGCGGCGATATCCATGGCCAGTTTTATGACCTAATTGAGCTATTTCGTA
TTGGTGGTAATGCTCCTGATACTAATTACCTCTTCATGGGAGATTATGTAGgtatg
tctttctctcttctctatcactctatttcaatttttttgtgtgggtaagttaaatttcctgatttgttgggtttttagagatctgtgttcttatgttg
attttcagagcatgtttgattcaaagtacgctggttcagtttgaagtttggagctaatagttatttctttggtttattgttgtcttggttatatc
tgtgcagATCGTGGCTACTATTCTGTAGAAACAGTCTCTCTATTGGTGGCATTAAA
GGTGCGTTACAGGGACAGACTTACGATCCTGCGAGGGAATCATGAGAGCCGT
CAGATTACACAAGTgtaagattttctttctaagaagacttgtatttcatttgcttctgactcgcaagcactaacaatggttt
gtgcctgtttttgcttatgtttgttcagCTATGGTTTTTATGACGAATGCTTGAGGAAATACGGAA
ATGCAAATGTGTGGAAGTATTTTACGGACCTTTTCGATTATCTCCCTCTTACA
2AC11→
GCACTCATAGAGAGTCAGgttcgagctgttcttgccagtcgtcattgtatcatccgtagttgtttcacttccgcacttt
tcttcatcaactgtattccgtgacagGTTTTCTGTTTGCATGGAGGCCTTTCACCTTCTCTGGAT
ACTCTTGACAATATCCGAAGCTTGGATCGAATACAAGAGgtattgttttgggtatagaagcat
tgttttgcatttagtatctgtaaatgtctcctctttttccctaaagacattgtttctctctggctataaactctctgaaaccaacctcctagtt
attattagttttggcttcagatagaacaaggattgtaataatctgatatggtatgctctgggattgtagGTTCCACACGAAG
GACCAATGTGCGATCTACTCTGGTCTGATCCCGACGATCGTTGTGGATGGGGA
ATATCTCCTCGTGGTGCTGGTTACACGTTTGGACAGGACATTGCTACTCAGTTT
AATCATAACAATGGACTGAGTCTGATCTCAAGAGCGCATCAACTTGTAATGGA
AGGCTATAATTGGTGTCAGgtatataatatgtgcataacaatagattggcatatgtgttgaatagggttctggttgc
aaaaatgctgaatctttcggtgtgttgatagGAAAAGAACGTAGTGACAGTGTTTAGTGCACCGA
ACTACTGTTACAGATGTGGAAACATGGCCGCAATTCTTGAGATTGGAGAAAA
GATGGAACAGAACTTCCTTCAATTCGATCCAGCACCTAGACAAGTCGAACCCG
c1-1
ATACCACGCGCA▼*AGACCCCTGATTATTTTTTGT**GA***tttcatttttttcttccaaagtttgtttgtt*
←2AC12
gctgtatcattgtagatgtgtctgttttatttgttttttcgagtctctagatggaatgtgataccaaagacgaaaaacccatcattttt
ttgttgatgttgatactgaaacaggtttgtagaagcctcttcttattatagaaaatgtcttttgata

Figure 6. Unspliced sequence of the *Arabidopsis* PP2A *C1* gene (At1g59830). The start and stop codons are in bold. Coding sequence is in capitals. The 5′ UTR and 3′ UTR sequences are in italics. The locations of T-DNAs predicted by SIGnAL are marked by ▼. The locations of T-DNAs re-sequenced by me are marked by *. The locations of primers are underlined and the orientation of primers are denoted by small arrows.

2AC21→

taaccgggtcataaaacatttatactttgtctatttggacacgacccacttttagtctttaaaaaaaaaaaaacgaatatccatttttttc

tcagccaggtgagagagagagacatcgaactatcgaagaccag▼*agaagctaggaggatttgatctctccgattcggat*

c2-1 ←2AC22

*at*cttagctctggccgatagattctccggcgattcatcttctttatccgccgtaattttcgtatataggatcggtggtggctttcaa*

g**ATG**CCGTCGAACGGAGATCTGGACCGTCAGATCGAGCAGCTGATGGAGTGT

AAACCGTTATCGGAGGCGGATGTGAGGACGCTTTGCGATCAAGCGAGAGCGA

TCCTTGTCGAGGAATATAATGTTCAGCCGGTGAAGTGTCCTGTTACCGTTTGC

GGCGATATTCACGGCCAGTTTTATGACCTTATTGAGCTCTTTCGTATCGGTGGC

AACGCTCCTGATACTAACTACCTCTTCATGGGAGACTATGTAGgtctgtctttcttcttctttt

cgattttatttttggggttttttacttttttttctcattttagatctgtgtttttggatcattccaaaatggtgaattcttggaaaagtatccaccttt

ttatttatctagagtgagttacttgtgggttctgtttttttttggaatggtaggtactgaaacttcccaatatagagacttgttgatgctcact

ttcattattgcacttcccttgaattctagtctttagatccttggaactttaaccattcgttggtgtaccataagatggtgacagctagattta

gtttaaaaggagtgttgttggtttctcattgtcttgctttgttctttgcagATCGTGGCTACTATTCAGTAGAGAC

AGTTTCTCTATTGGTGGCACTAAAAGTGCGATACAGGGATAGACTTACAATCT

TACGAGGGAATCACGAGAGTCGGCAGATTACTCAAGTgtaagcttttgttttgaaataagcttat

atttgagcgaatcgtacttttcttcattggtttctggctgatcagcaaagtactatcctgttttgatgcatgttttgcttctctgtttcagCT

ATGGTTTTTATGACGAATGCTTGAGGAAGTACGGAAATGCTAACGTCTGGAAG

TATTTTACAGACCTTTTCGATTATCTTCCTCTTACCGCCCTCATAGAGAGTCAG

gttagagctgttctttctcagttggtgtttgatacatctttttctaagtacagctttttgacttcatcaaattttgtagGTTTTCTGTT

TGCATGGAGGGCTTTCACCTTCTCTGGATACTCTTGATAATATCCGAAGCTTG

GATCGGATACAGGAGgtaaccttcttgttctctgctaaaggccttagtgtggacttagttggaagagttatgagtccttc

ctgggtctcagtgtcttgtctctacttaagatacaattacaaagagcacaaacgtttggaatttttatgacatcttattgttcagctctttg

aactatgtatgatgtgtgatctgtagGTTCCACACGAAGGACCTATGTGTGATTTATTATGGTC

TGATCCTGATGATCGATGTGGATGGGGAATATCTCCACGAGGTGCTGGTTATA

CATTTGGACAGGATATCGCAGCTCAATTTAATCACAACAATGGACTAAGTCTC

ATATCAAGAGCGCATCAACTTGTCATGGAAGGTTTTAACTGGTGTCAGgtatgaac

attcttacggcatgattagtctctctctcagattgaatcgttgatctctcctttgtatggtaacagGATAAGAATGTGGTG

ACTGTGTTTAGTGCACCAAACTATTGCTACCGGTGTGGAAACATGGCTGCCAT

TCTAGAGATAGGAGAGAACATGGAGCAAAACTTCCTCCAGTTCGATCCAGCT

CCTCGACAAGTTGAACCTGATACTACTCGGAAGACCCCTGATTATTTTTTGT**G**

A*tttctctgttttttttgtggtcccgaccatatataatttttgaagttccagttttattgcctatgtatcattgtagatgtgccctttctct*

tctttttctaagtggagttcgaatttcccctagaaaaaaagaaacacatcatttttttctgttgttgaggtgattgctataaaaaagt

ttgtaggagaggcttctatatctataaaattgagcgtttgtctt

Figure 7. Unspliced sequence of the *Arabidopsis* PP2A *C2* gene (At1g10430). The start and stop codons are in bold. Coding sequence is in capitals. The 5′ UTR and 3′ UTR sequences are in italics and the remaining genome sequence is in lower case letters. The locations of T-DNAs predicted by SIGnAL are marked by ▼. The locations of T-DNAs re-sequenced by me are marked by *. The locations of primers are underlined and the orientation of primers are denoted by small arrows.

2AC35→

AAATTCATCCCGAGAAATCGAAGTTTTCCCCGACCCAATACTTTCACCGGTATCAAATCTCGTCGCCATcacaga

c3-3 2AC31→ *c3-2*→

gataaaaaagagagagcgagactgtgagactagagagagaga*gcttta▼tgaagcgaaggcaaaagtctctcgagacgattatataag▼*ctgaaaccggttcagttaaac
cgtagaagttagtattaaaccggtgatggctattgcaccaattaaagtagaggggtattatggtaatagcaaaagaaagagctcgaaagatacaaaccactcgcttctctcctcacgc
gccttctcaaaattcgatcttctttcactgcacacaaagc*gaagagacaaagaagagagagagagagagagagagagagagagagagagagagagagaaagtgggggg*
aaaaaaggcgaagaatacgcagattccatccaatttagggttaggacttcccctaatttggaaacctagtcctgtttcctgatctgttttccggatcggacgagagacgaattaa
*cgaaa***ATG**GGCGCGAATTCTATTCCGACGGACGCAACCATTGATCTTGATGAGCAGATCTCGCAGCTCATGCA
GTGCAAGCCTCTCTCGGAGCAACAGgtgcctacaactttctctttagaccttagcccttgacgttttctctactttgttttttatcgatgattggatctgttttttttgttttt

c3-1

ttgagtggagtggtatttttttctgtgtttgaggtttcttgggtacgagattatcagatttgatttggaaagtttcgatttttttttttttttct*aaatgaacatcttcaggaaatgttattagatctt

←2AC36

g▼gcatgtatgttctcacggaagctcatttgtcaccgcagttctatgggttggagaattggctttgcttctatggtttgatgttaaatttggctgatttggattcttagcatagtgattaatc

←2AC32

acatctaagctcgtgtttttatgtgcctggtaatgtcaatcgggattcttatggactgataaatttttaatattttcgtatcaatagctgatcaatacttgttggtttttttttttcttaattgatgctc
ctgtttgttgcctgtgtcaagttgatttctctaagtatatatttctatgtcatgtgtttgatagtggttattagctgcatgtcttgagagtgttaaagaggtgaaaatcatatgcagcattgttttat
tttccttagGTTAGAGCATTATGCGAGAAAGCCAAGGAGATCTTAATGGATGAAAGCAATGTTCAGgttagttcctggtcttg
catatctctttggttgagcaatttctttcaccacacttggtgattctattttcagtgtggtagattgctctactagccatgaatttattagttctttctatttcctgcatgcagCCTGTGAA
AAGCCCTGTGACAATCTGCGGTGATATTCATGGACAGTTCCATGATCTTGCAGAGCTTTTCCGTATAGGGGG
AATGgtaacatctataaaatatacagagctctacaaatagctctaccaatcttacaaagtattaattttctctttttgcagTGCCCTGATACCAATTACCTGTTT
ATGGGAGACTATGTGGACCGTGGTTATTATTCTGTTGAAACTGTTACGgtacgaagatgaaatatttagacttccttttttattcatttaa
gagtaggctgattggcaagccagtcattgtctttttatattggtaacaagataccattaacttgacggatatttttttctttacaatgtatatattttgggtgtgactatgaagtatgctgatact
gtttctctttcttattctctttccagCTGTTAGTCGCCTTAAAGATGCGATATCCTCAGCGAATCACTATTCTTAGAGGAAACCA
TGAAAGTCGTCAGgtagactaatgctttatgattttattactctggttccacttgtaaattcaccttctgatgttaaatacagtacttcattgtgtatccagtcttttccatcgttga
gatcttggttgacacacaatttccttttttataagtctctgttttgtctacaaaattcgttttgtgcacatttttttttatcatattactatttaatctgacacaggcttttcttctttttcttccatctcgc
agATTACTCAGGTTTATGGATTTTATGATGAATGTCTACGAAAgtgagtctcctggaaaaaatcctatactggaaaaaaaatccttatttttcta
atgcatttaaacaatttttttaattattattagcattactattgtccttatatttttgctgttgcattgtatgtgtgtatggtagcctgtccaaaattgtcttttcttttcttttacttcctgtttctgttaatcc
tttgggcttatgattacttgcttatagtattgttctatgttgtataatcttacacatcctagtttgcagcttatttgggatatagattttgcaataaatttaacaaaccatgttttccttttttagGTA
CGGCAACGCAAATGTTTGGAAAATCTTTACAGACCTCTTCGACTATTTTCCTCTGACAGCCTTGgttagtacctatcata
catgtctcacatttttcttcctttttttgttagtgggggttcacatataagtctatcctcgtccggtagtcaattaatatagatgatcagttctttgtcttgtgctggcagtattatttggaaccaaa
gtagtacttttgtactgtgctgtcgtatttctttgctagtgtcacatcaattgcaagacgttagtattctgaaaaatgcaaagaatggagatctgttatatatggaagatttcatgtatgcctt
atctgactaaatcgttggtatttttctacacaaatttttgggaatcatcaaatcattatgtttttttccctgcagGTTGAGTCAGAAATATTTTGCCTTCATGGTG
GATTATCTCCATCTATCGAGACCCTTGACAACATAAGGAATTTTGATCGAGTTCAAGAAGTGCCCCATGAAG
GGCCGATGTGTGACTTATTATGGTCTGACCCCGATGACCGATGTGGTTGGGGCATCTCTCCTCGGGGTGCCG
GATATACATTTGGTCAGgtaattacaagtcttacgtctcagaaacagtcctgattctctctgtttttaaagcaagtgtcgaattgacttttctcttttttcattttgtttccatag
GATATATCTGAACAATTCAATCACACAAACAACTTAAAGCTGATCGCCCGAGCTCACCAGTTGGTTATGGAT
GGATACAACTGGGCTCACgtaataatgaaactataaaccttttctttgcatagctttgcaaggcttaagattctgagctcatttttactgtcttccctatcaaacagGAG
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cttgagtatataaaatgtgagtgcagTTTGAACCAGCACCGAGGAGAGGAGAACCAGACGTCACCCGAAGGACTCCAGACT
ATTTCCTG**TGA***tcactggacacagactcatctcaatcagcagctgcaggcacccacaagtgaaaagtcacagttggctaactctagattctggtcctatgcattgtgtt*
caggttgctctgtttaaaattcaacttagtgcccaccaccggaatatagtgtgtattataggcgaagaaggcttcttatcttcctctataaccaagaaaagaaaaaagatacccca
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aatagaaccttgaaatgttatgtttatttgttcttcaaagcttgatataataacaatagtcgctgaacgcatcagatcagccactcaaa

Figure 8. Unspliced sequence of the *Arabidopsis* PP2A *C3* gene (At2g42500). The start and stop codons are in bold. Coding sequence is in capitals. The 5′UTR and 3′UTR sequences are in italics and the remaining genome sequence is in lower case letters. The locations of T-DNAs predicted by SIGnAL are marked by ▼. The locations of T-DNAs re-sequenced by me are marked by *. The locations of primers are underlined and the orientation of primers are denoted by small arrows.

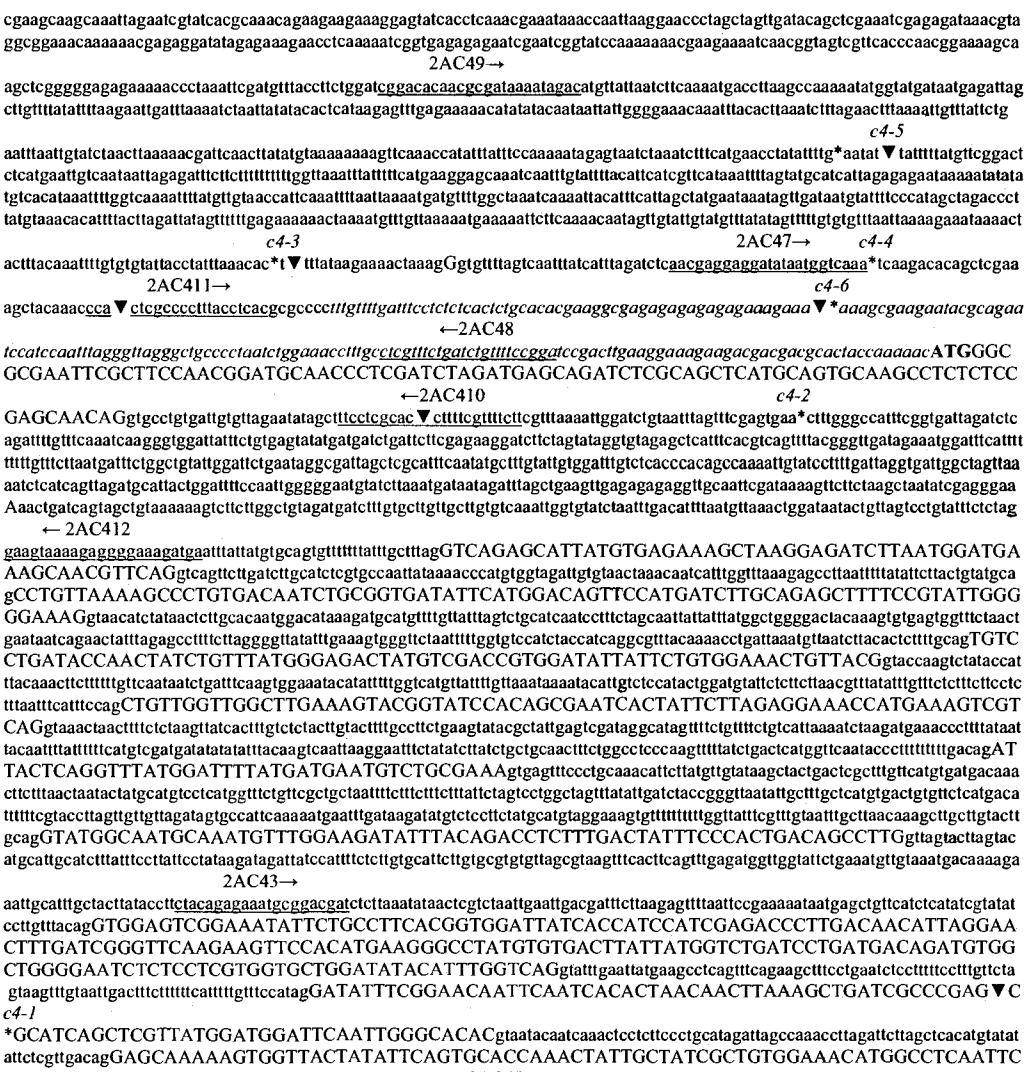

Figure 9. Unspliced sequence of the *Arabidopsis* PP2A *C4* gene (At3g58500). The start and stop codons are in bold. Coding sequence is in capitals. The 5′UTR and 3′UTR sequences are in italics and the remaining genome sequence is in lower case letters. The locations of T-DNAs predicted by SIGnAL or GABI are marked by ▼. The locations of T-DNAs re-sequenced by me are marked by *. The locations of primers are underlined and the orientation of primers are denoted by small arrows.

gtaactgaagaccagtcttaaagctggaacatttgaactaaacatatacgactgctcctatcgctctagaaatttaattaacaattattcatagttact
taattacaaagacaacaataaaggtgaacgaataaattaaaggaaaattatgaaaatagtttaatttgtatgaaacttttcatccgcataccaaatgt
2AC511→
catatactcttatcttacatttttctcttgggacatatttttggagcgaacactttcgcatgtacgataaggttcagtggtctgagcttcgactacgttaa
c5-1
aatgatgattagggtttactaatttggaattaggagttacatttaaggttaaaagtttatgagtttcaagtttcaac▼*gaagctttaaagtttaaacg
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←2AC512
*caaacacaaaccggatccgagattaaaaacaatccggattgtttatatttactttagatcggagaaag***ATG**CCGCCGGCGAC
CGGAGATATCGATCGTCAGATCGAGCAGCTTATGGAGTGTAAAGCGTTATCTGAAACG
GAGGTGAAGATGTTGTGTGAGCACGCAAAGACGATTCTTGTGGAAGAGTATAATGTTC
AACCGGTTAAATGTCCGGTTACCGTCTGCGGTGATATCCACGGCCAATTTTACGATCTA
2AC513→
ATCGAGCTTTTTCGTATCGGTGGTTCTTCTCCTGATACTAATTATCTTTTCATGGGTGAT
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tttgtcattgaaaagacgaaacttttgttttagctgttaactattctcagggcttctacaatggagtagcttgaccttgggagatataacattttaatgta
c5-2
gtttgtgaagcttcatgttcgagtactcggatttgtgtttctcttttctctgt▼*ttaagtgccttaagctccgtttcctacttaagttgctcaattagcta
actaatggatcattgttaaacttgtgaagATCGAGGGTATTATTCTGTGGAGACAGTCTCACTTTTGGTAG
CACTGAAAGTTCGTTACAGAGATAGACTTACTATCCTAAGAGGGAATCATGAAAGCCG
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TTACATGGAGGACTTTCACCTTCTTTAGATACACTTGACAACATCCGATCTTTAGATCG
AATTCAAGAGgttagttccagaatccaaaccaacaaaaggatgcaccttacttgatgtttacataacgagactaagatgtttgtgttgcct
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CGGTTGGGGAATATCTCCTCGTGGTGCAGGCTACACTTTCGGACAAGATATCGCTACT
←2AC514
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TGCTACCGTTGTGGCAACATGGCTGCGATTCTAGAGATCGGTGAGAACATGGACCAGA
ATTTCCTTCAGTTTGATCCAGCTCCACGTCAAGTCGAACCCGAAACCACTCGCAAGACT
CCAGATTATTTTTTGT**TAA***gtaccaaaagaactaaagctgcaattccacacactagctcttcgtctctttgcttctattcttaacc*
acttttttgtaatttcatattccttacgtctatttctgtttgttaaactctccattgtcttttaaaatccgtaatttttttgtgtgcaaagattctggtccaagt
aactgaaaccgctatcacc

Figure 10. Unspliced sequence of the *Arabidopsis* PP2A *C5* gene (At1g69960). The start and stop codons are in bold. Coding sequence is in capitals. The 5′UTR and 3′UTR sequences are in italics and the remaining sequence is in lower case letters. The locations of T-DNAs predicted by SIGnAL are marked by ▼. The locations of T-DNAs re-sequenced by me are marked by *. The locations of primers are underlined and the orientation of primers are denoted by small arrows.

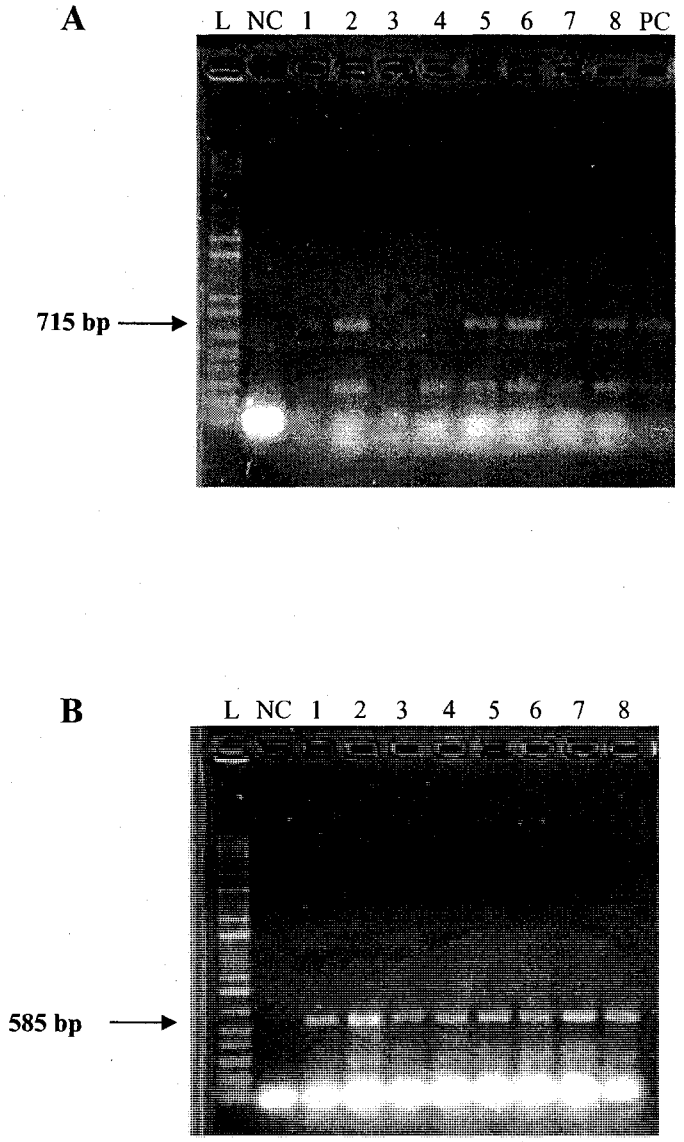


Figure 11. PCR identification of plants homozygous for the mutant *c4-1* allele. **A.** PCR amplification of wild type copies of the PP2AC-4 gene with primers 2AC43 and 2AC44. The expected size is 715 bp. Lane L contained a size marker. Lane NC (negative control) contained no template DNA. Lanes 1-8 contained template DNA from individual kanamycin-resistant plants. Lane PC (positive control) contained wild type DNA template. Arrows indicate plants with no PCR product. **B.** PCR amplification of T-DNA product with genomic primer 2AC43 and T-DNA primer JMLB1. The expected size is 585 bp. Lane L contained a size marker. Lane NC (negative control) contained no template DNA. Lanes 1-8 contained template DNA from individual kanamycin-resistant plants. The product at ~ 300 bp is likely to be due to non-specific primer binding.

produced a PCR fragment with the expected size indicating that the primer set could synthesize a product of the expected size (Figure 11A, Lane PC). The second positive control used primers 2AC43 and JMLB1, which is located within the T-DNA left border, and produced PCR fragments with the expected size of 585 Kb from all 8 plants, indicating that the template functioned in PCR (Figure 11B) and that all plants had a T-DNA insertion in the *C4* gene as expected since they germinated on kanamycin. I conclude that the negative PCR results in Figure 11A with the two PP2A C4-specific primers are due to homozygosity of the gene for the T-DNA insertion. The seeds were collected from these homozygous mutant lines and used for further analysis.

I have obtained homozygous mutant lines for all five C subunit genes (Table 4). Seeds for only one T-DNA insert in the *C1* gene were available at ABRC. A second *C1* insertion line was obtained from GABI but did not germinate. For the *C2* gene, multiple lines were available from SALK, but only one germinated. Multiple homozygous lines were obtained for the other three C subunit genes. When it was discovered that only one *C4* mutant line showed a phenotype (details in Chapters III and IV), another *C4* mutant line with allele number *c4-2* was ordered from GABI to confirm this phenotype (Table 4).

Identification of T-DNA Insertion Sites in the PP2A C Subunit Genes

Although the sites of the T-DNA insertions in the *Arabidopsis* genome had been identified by the Salk Institute Genome Analysis Laboratory (SIGnAL) and by GABI and the data are available via the T-DNA Express website or GABI website, sometimes the information is not completely accurate. Inaccuracies in placement of the

Table 4. Homozygous PP2A T-DNA insertion lines. Differences between predicted and confirmed T-DNA location (based on cDNA, EST data and my sequencing results) are in bold.

Subunit	Gene Identifier	Allele Number	Predicted T-DNA Insertion Location	Confirmed T-DNA Insertion Location
C1	At1g59830	*c1-1*	6th of 6 exons	6th of 6 exons
C2	At1g10430	*c2-1*	5′ UTR	5′ UTR
C3	At2g42500	*c3-1*	1st of 10 introns	1st of 10 introns
		c3-2	**5′ UTR**	**Promoter**
		c3-3	**5′ UTR**	**Promoter**
C4	At3g58500	*c4-1*	9th of 11 exons	9th of 11 exons
		c4-2	1st of 10 introns	1st of 10 introns
		c4-3	Promoter	Promoter
		c4-4	**5′ UTR**	**Promoter**
		c4-5	Promoter	Promoter
		c4-6	5′ UTR	5′ UTR
C5	At1g69960	*c5-1*	Promoter	Promoter
		c5-2	1st of 4 introns	1st of 4 introns

T-DNA may arise either from misidentification of the gene region where the T-DNA is localized (for example, the 5′ UTR is difficult to predict without cDNA or EST sequences) or from poor quality sequence data due to the high-throughput nature of the T-DNA sequencing effort. With more 5′ and 3′ EST data, as well as full-length cDNA sequences available, the region where the T-DNA inserted can now be defined more accurately. Based on EST and cDNA sequence data, I have defined the 5′ and 3′ untranslated regions (UTR) and coding sequences for all five PP2A C subunit genes (Figures 6-10). PCR fragments containing the T-DNA left border insertion junction were amplified with one T-DNA primer (usually JMLB1) and the appropriate PP2A C subunit gene primer. The PCR products were then sequenced with the PP2A C subunit gene primer. The sequences were aligned with the genomic PP2A C subunit sequence using the SeqMan program from the DNAStar package (Lasergene, Madison, WI).

I have identified a total of 13 T-DNA insertion alleles from the five PP2A C subunit genes in *Arabidopsis*. Ten of the insertion sites exactly matched the gene region predicted in the sequence databases at SIGnAL and GABI. However, in three of these alleles, *c3-2, c3-3* and *c4-4*, the region where the T-DNA is localized was incorrectly predicted. In all 3 cases, the T-DNA is in the promoter region rather than the 5′UTR as indicated in the sequence database (Table 4). My sequencing results showed that the location of the T-DNA in most alleles was within 6 bp of the location indicated by the database. However, in four cases, the T-DNA location differed by between 30 bp to 53 bp from the location indicated by the database. These results are shown in Figures 6 -10 and Table 5.

Table 5. Comparison of the predicted and actual locations of the T-DNA insertions in the PP2A C subunit genes. See Figures 6-10 for sequence data. Positive values indicate that the actual insertion site was downstream of the predicted site, negative values are upstream.

PP2A Subunit	Gene Identifier	Allele Number	Base pairs Different Between Predicted and Confirmed Insertion Site
C1	At1g59830	*c1-1*	0
C2	At1g10430	*c2-1*	37
C3	At2g42500	*c3-1*	-35
		c3-2	0
		c3-3	-6
C4	At3g58500	*c4-1*	1
		c4-2	53
		c4-3	-1
		c4-4	-30
		c4-5	-5
		c4-6	0
C5	At1g69960	*c5-1*	0
		c5-2	0

DISCUSSION

The purpose of the experiments described in this thesis was to identify the function of PP2A C subunits in *Arabidopsis* with a reverse genetics approach. In contrast to forward genetics, reverse genetics starts with a mutant gene and attempts to identify a phenotype or phenotypes caused by this mutation. The Salk Institute Genome Analysis Laboratory (SIGnAL) created a sequence-indexed library of T-DNA mutations in the *Arabidopsis* genome (Alonso et al., 2003). T-DNA insertion sites were determined using TAIL-PCR and posted on their website. In selecting the T-DNA insertions to use for my research, I purposely did not choose alleles in which the T-DNA had inserted in the 3′UTR since there is a good chance that a full-length coding sequence might still be transcribed from such alleles. If mRNA or protein can subsequently be produced, these T-DNA insertions are not likely to disrupt gene function.

I used kanamycin resistance and PCR to identify homozygous lines for all five PP2A C subunit genes. Segregating T_3 seedlings which grew in germination medium with kanamycin should have T-DNA inserts in their genome and seedlings which died should have no T-DNA inserts in their genome. However, in some cases, after being grown on the kanamycin-containing germination medium, all of the seedlings were dead. This happened both in T_3 generation seeds ordered directly from ABRC and in seeds collected by me after several generations. If seeds were germinated in medium without kanamycin, all seedlings looked healthy as expected. PCR showed that about 25% of the seedlings were genotyped to be wild type for the PP2A gene; about 25% of the seedlings

were homozygous for the T-DNA insertion in the PP2A gene; and about 50% of the seedlings were genotyped to be hemizygous for the T-DNA insertion in PP2A gene. These results matched the predicted ratios for segregation of a single T-DNA insertion. One explanation for the loss of kanamycin resistance is that after several generations of growth, the kanamycin resistance gene may be silenced leading to loss of the drug resistance phenotype. Gene silencing can happen at the transcriptional level. For example, histone modification creates an environment of heterochromatin around a gene that makes the promoter inaccessible to the transcriptional machinery such as RNA polymerase and transcription factors (Hawkins and Morris, 2008; Vaissiere et al., 2008). Gene silencing also could happen at the post-transcriptional level resulting in the mRNA of a particular gene being destroyed. The destruction of the mRNA prevents translation to form functional protein (Hawkins and Morris, 2008). Although silencing of the kanamycin resistance gene interferes with the ability to use antibiotic selection to identify plants that retain the T-DNA, silencing does not affect the ability of the T-DNA to inactivate the gene where the T-DNA inserted. Thus, PCR is a good approach for identifying homozygous mutants since it is independent of the antibiotic resistance phenotype.

Sometimes, the T-DNA insertion site in the genome is inaccurately predicted by the database. Therefore I sequenced all of the alleles to identify exactly where the T-DNA insert was located. My sequencing results showed that, in most alleles, the T-DNA insert was very close to the place indicated by the database except for the *c2-1, c3-1, c4-2* and *c4-4* alleles. In these four cases, the T-DNA inserts were more than 30 base pairs

away from the insertion site indicated by the database. The insertion sites of the alleles I worked on are located in the promoter region, 5′ leader, exons or introns. These alleles have the best potential to be knockouts. The next step is to investigate whether the T-DNA insertion disrupts the expression of the PP2A C subunit gene.

CHAPTER III

CHARACTERIZATION OF PROTEIN PHOSPHATASE 2A C SUBUNIT MUTANTS IN *ARABIDOPSIS THALIANA*

Introduction

T-DNA mutagenesis of *Arabidopsis* can produce several consequences and these have been named "knockout", "knock-down", "knock-on", "knock-about" and "knock-knock". The effects on gene expression include reduced expression, increased expression and no expression (Krysan et. al., 1999). A knockout is the most desirable outcome of the insertion since the gene is effectively inactivated and unable to produce a functional gene product. T-DNA is a large DNA fragment (usually >5 Kb) and, when inserted into an *Arabidopsis* gene, it is expected that no message or a truncated message will be formed. In a knockout, the sites of T-DNA insertion are usually in the promoter region, 5′ UTR or coding region as well as in introns (Krysan et. al., 1999). However, T-DNA mutagenesis of *Arabidopsis* does not always disrupt gene expression. For instance, in a knock-about, if T-DNA inserts in the promoter or 5′ UTR and the T-DNA contains a promoter oriented toward the gene, a partial transcript and thus a functional message could be formed. Thus, identifying a true knockout plant is essential for conducting screens for mutant phenotypes.

One technique to determine if a T-DNA insertion has altered gene expression is reverse transcription-polymerase chain reaction (RT-PCR). Since the majority of my inserts are in the 5′UTR (Table 4), I wanted to be sure that there was no downstream expression because insertion in the 5′ UTR can produce a truncated message that contains the entire coding sequence if there is a functional promoter in the T-DNA in the correct orientation. Two primers were designed to bind specifically to the PP2A sequence at a location downstream of the T-DNA insert. If no message is produced, no product will be observed after RT-PCR.

After confirming that the T-DNA insertion interrupted the PP2A C subunit genes, the next step is to determine the effects of the mutation on plant growth and development relative to the wild type. Previous pharmacological approaches using the PP2A inhibitor okadaic acid have revealed that PP2A regulates a variety of cellular functions including ion channel activity (Li et al., 1994), cytoskeletal organization (Smith et al., 1994; Foissner et al., 2002), carbon and nitrogen metabolism (Siegl et al., 1990; Huber et al., 1992), and cold responses (Monroy and Dhindsa, 1995). Although inhibitors have been very helpful for assessing the cellular functions of PP2A in plants, the specific PP2A heterodimers or heterotrimers involved cannot be fully explored without using genetic approaches.

To date, three PP2A mutants have been identified in *Arabidopsis* using genetic approaches. *rcn1* was the first PP2A mutant identified. RCN1 encodes the PP2A A1 subunit. As described in Chapter I, RCN1 participates in signaling pathways for several plant hormones including auxin, ethylene and abscisic acid (Garbers et al., 1996; Deruere

et al., 1999; Rashotte et al., 2001; Kwak et al., 2002; Larsen and Cancel, 2003; Michniewicz et al., 2007). Data from a dephosphorylation assay showed that the *rcn1* or *a1* mutant seedlings contained lower levels of phosphatase activity than wild type plants indicating that the phenotype was due to an enzyme deficiency (Deruere et al., 1999). Furthermore, PP2A *a1a2* and *a1a3* double mutants exhibited extreme abnormalities in all stages of development (Zhou et al., 2004).

The second identified PP2A mutant was *ton2* which was isolated in a screen for *Arabidopsis* mutants affected in cell elongation (Camilleri et al., 2002). As I mentioned in Chapter I, since the TON2 amino acid sequence had a very low level of amino acid sequence similarity (<20%) with other B″ subunits from plants and animals and no similarity to B or B′ subunits, it might be a new type of PP2A subunit which doesn't fit with the B″ group.

A third PP2A mutant phenotype was identified in plants carrying a disrupted PP2A C2 subunit. Evidence from phenotypic, genetic and gene expression data strongly suggest that PP2Ac-2 is a negative regulator of the ABA signaling pathway (Pernas et al., 2007).

In this study, I used RT-PCR to investigate whether PP2A transcript was absent in the homozygous T-DNA mutant lines that I identified for the PP2A C subunit genes. After identifying five bona fide knockout mutants, I next focused on identifying and characterizing any hormone and stress response phenotypes associated with the homozygous PP2A C subunit mutants.

Materials and Methods

Growth of *Arabidopsis*

Arabidopsis thaliana was grown in liquid culture for RNA isolation. MS medium contained 0.44% (w/v) Murashige & Skoog basal salt mixture with vitamins (Caisson Laboratories, Inc., North Logan, UT, Cat. No. MSP0506), 0.05% (w/v) MES and 1% (w/v) sucrose. Medium was adjusted to pH 5.7 with KOH and 50 ml of medium was autoclaved in 125 ml glass flasks capped with aluminum foil. Seeds were sterilized by bleach treatment. Seeds were placed in a 1.5 ml microcentrifuge tube and incubated for one minute with 70% (v/v) ethanol. The ethanol was removed and the seeds were incubated with occasional agitation for 12 min in a freshly-made solution of 30% (v/v) Clorox bleach containing 0.1% (v/v) Triton X-100. Under sterile conditions, the bleach was removed and seeds were washed at least 5 times with 1 ml sterile water each time, until the solution did not foam when shaken vigorously. Typically 20-50 seeds were used per flask and sterile seeds were added directly to the medium. Flasks were placed on a platform shaker at about 150 rpm in a growth chamber (22°C, 18 hour light cycle).

RNA Isolation

Two-week-old plant tissue was rinsed, blotted dry, frozen and stored at -80°C until extraction. Plant tissue (0.5 mg) was ground in liquid nitrogen using a mortar and pestle. TRIZOL (5 ml; Invitrogen, Carlsbad, CA) was added to the plant tissue, vortexed and incubated at room temperature for 5 minutes to permit dissociation of nucleoprotein

complexes. The sample was centrifuged at 10,000 x g for 10 minutes at 4°C to remove insoluble material from the homogenate. The cleared supernatant was transferred to a fresh tube and 1 ml of chloroform was added and shaken vigorously for 15 seconds. After incubation for 2 to 3 minutes at room temperature, the sample was centrifuged at 10,000 x g for 15 minutes at 4°C. The aqueous phase containing the RNA was moved to a new tube and 2.5 ml isopropanol was added and mixed. The sample was incubated for 10 minutes at room temperature and then centrifuged at 10,000 x g for 10 minutes at 4°C. The supernatant was removed and the pellet was washed with 5 ml 75% ethanol. The sample was centrifuged at 10,000 x g for 5 minutes at 4°C. The pellet was air-dried for 10 minutes and resuspended in 400 µl of DEPC-treated water. The RNA sample was stored at -80°C.

Primer Design for RT-PCR Analysis

Primers specific to the five PP2A C subunit genes were designed using either Primer Design (Lasegene, Madison, WI) or Oligo 4.0 (National Biosciences, Plymouth, MN). All primers were synthesized by Integrated DNA Technologies (IDT). The primers were designed in exons and had at least one intron between primer pairs when possible.

RT-PCR

RT-PCR was performed on a PTC-100 programmable thermal controller (MJResearch, Watertown, MA). Each reaction consisted of 2.5 µl of 10x Taq buffer (BD Medical, Franklin Lakes, NJ), 2.5 µl of 0.1 M DTT, 0.85 µl of 1 M KCl, 0.5 µl of 10 mM dNTPs, 0.66 µl of both the forward and reverse primers at 1 pmol/µl, 0.5 µg of RNA, 0.5

μl of 50x Taq DNA Polymerase (BD Medical, Franklin Lakes, NJ), 20 units of RNAsin (Promega, Madison, WI) and 25 units of SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). The reaction was brought up to its final 25 μl volume with DEPC-treated water. Reactions were performed according to the following program: 45°C for 60 minutes for cDNA synthesis, followed by 94°C for 5 minutes and then 36 cycles at 94°C for 30 seconds, 52°C-70°C for 30 seconds depending on the primers' annealing temperatures (Table 6) and 72°C for 2 minutes. The program finished with 72°C for 5 minutes.

Phenotype Screening

In this study, I focused on plant hormone responses and stress responses using protocols obtained or modified from the *Arabidopsis* Gantlet Project website (http://thale.biol.wwu.edu/) and from the literature. Methods listed in Chapter II are not repeated here. The details of each phenotype screening protocol are listed below. Each screen was done using two or three plates of each genotype for a total of 15-25 seedlings.

Root Curling Assay

Plates for this assay contained Low Sucrose MS medium. Fifteen wild type seeds were surface sterilized and plated in a row on the left side of the plate. Fifteen PP2A mutant seeds were surface sterilized and plated on the right side of the plate. After stratification, the plates were placed horizontally under standard growth conditions for eight days. On day 8 the plants were photographed and root curling on the bottom of the plate was observed.

Table 6. Primer sequences used in RT-PCR.

Primer Name	Primer Sequence (5′→3′)	Annealing Temperature (°C)
2AC1NPU	GCTGTATCATTGTAGATGTGTCTG	68
C1RTPCRUU1	GATTCGGTGGCTATTGGTTG	60
RTPCR-C1102599-L-2	ACCTGTTTCAGTATCAACAT	54
RNAs150673-F	TGGCAACGCTCCTGATACTAACTA	70
RNAs150673-R	AAAAAGAAGAGAAAAGGGCACATC	66
RTPCR/C3U	GCATTATGCGAGAAAGCC	54
2AC3NPL	GGCTGATCTGATGCGTTCAG	62
RTPCR/C4s035009U	CCAAACTATTGCTATCGC	52
RTPCR/C4s035009L	TGATGCAGAAGGGAGCT	48
RTPCRC4-2S-F-2	AAGACGACGACGCACTACCAAAAA	70
RTPCRC4-2S-R-2	GATGCAGAAGGGAGCTAATAAGTT	68
RNAs013178-F	TGTATGGGTTTTATGATGAATGTT	62
RNAs013178-R	AATAGAAGCAAAAGAGACGAAGAG	66
2AC513	TCGGTGGTTCTTCTCCTGATACT	66
ACT1	GGTAACATTGTGCTCAGTGGTGG	70
ACT2	CTCGGCCTTGGAGATCCACAT	66

Root Waving Assay

Plates for this assay contained Low Sucrose MS medium made with 1.5% agar (Phytoblend, Caisson Labs., Inc., North Logan, UT). Eight sterilized wild type seeds were plated in a line on the left side of each plate and eight seeds of a PP2A mutant were plated in a line on the right side. After stratification, plates were moved to standard growth conditions. The plates were tilted so that the top surface of the agar was at a 60° angle from horizontal. On day 5, the plants were photographed and root waving was observed.

Germination Assay

Plates for this assay contained Low Sucrose MS medium. Nine sterilized wild type seeds were plated on the left side of each plate and nine seeds of a PP2A mutant were plated on the right side. After stratification, plates were placed horizontally in standard growth conditions. After three days, germination was determined by recording the presence of a radicle using a dissecting microscrope.

Growth on Vertically Oriented Plates

Plates for this assay contained Low Sucrose MS medium made with half strength MS. Eight sterilized wild type seeds were plated on the left side of each plate and eight seeds of a PP2A mutant were plated on the right side. After stratification, plates were moved to standard growth conditions and placed vertically. After 5 days, seedlings were photographed and visually compared for differences in root growth direction between wild type and mutant plants.

Growth on Vertically Oriented Plates Followed by 90° Rotation

Plates for this assay contained Low Sucrose MS medium made with half strength MS. Six sterilized wild type seeds were plated on the top half of each plate and six seeds of a PP2A mutant were plated on the bottom half. After stratification, plates were moved to standard growth conditions and placed vertically. After 4 days, plates were rotated 90° counterclockwise and grown for another 4 days. The plates were photographed and visually compared for differences in root growth direction between wild type and mutant plants.

Growth on Medium Containing Ethanol

Plates for this assay contained Low Sucrose MS medium. After autoclaving and cooling the medium, ethanol was added to a final concentration of 0.5% (v/v). Nine sterilized wild type seeds were plated on the left side of each plate and nine seeds of PP2A mutants were plated on the right side. After stratification, plates were moved to standard growth conditions and oriented vertically. On day 6, germination was determined by looking for the presence of a radicle.

Growth on Medium with Additional Sucrose

Plates for this assay contained MS medium made with half strength MS plus 2% sucrose. Six sterilized wild type seeds were plated on the left side of each plate and six seeds of a PP2A mutant were plated on the right side. After stratification, plates were moved to standard growth conditions and placed vertically. After 5 days, seedlings were

photographed and visually compared for differences in root length between wild type and mutant plants.

Growth at 15°C

Plates for this assay contained Low Sucrose MS medium. Eight sterilized wild type seeds were plated on the left side of each plate and eight seeds of a PP2A mutant were plated on the right side. After stratification, the plates were moved to light for twelve hours daily at 15°C for 14 days. Plants were visually compared for differences in growth rate.

Growth on Medium Containing ACC

Plates for this assay contained ½ MSNS (MS medium without sucrose). After autoclaving, 1-aminocyclopropane-1-carboxylate (ACC; Sigma, Cat. No. 07626, St. Louis, MO) was added to the medium at a final concentration of 25 μM. Seven sterilized wild type seeds were plated on the left side of each plate and seven seeds of a PP2A mutant were plated on the right side. After stratification, plates were moved to standard growth conditions and oriented horizontally. After 24 hours, plates were wrapped in aluminum foil and placed vertically at 25°C, in the dark for another 4 days. After 4 days, the plants were photographed and visually compared for differences in hypocotyl length between wild type and mutant plants.

Growth on Medium Containing ABA

Plates for this assay contained ½ MSNS medium. After autoclaving, abscisic acid (ABA; Sigma, Cat. No. A1049, St. Louis, MO) was added to the medium at a final concentration of 1 μM. Fifteen surface-sterilized wild type seeds were plated on the left side of each plate and fifteen surface-sterilized PP2A mutant seeds were plated on the right side. After stratification, plates were moved to standard growth conditions and placed horizontally. After 3 days, seedlings were scored for germination by recording the presence of a radicle.

Results

Analysis of Expression of PP2A C Subunit Genes by RT-PCR

As described in the introduction to this Chapter, T-DNA can cause a knockout by disrupting a gene in such a way that no message or a truncated message is formed. In this study, RT-PCR was used to detect transcripts in plants homozygous for PP2A C subunit mutations. The C subunit is one of the most conserved proteins among eukaryotic organisms but the sequence of the 3′ UTR is more diverse. Two primers were designed to bind specifically to the PP2A gene sequence at a location downstream of the T-DNA insert. In order to make primers specific for each PP2A C subunit gene, I designed most reverse primers located in the 3′ UTR. If no message is present, no product will be observed in the RT-PCR. RT-PCR primers and the expected product size for each C subunit mutant allele are summarized in Table 7. Primer sequences are marked on the spliced sequence of each PP2A C subunit gene in Figures 12-16.

In a knockout, the sites of T-DNA insertion are usually in the promoter region, 5′ UTR or coding region as well as in introns (Krysan et. al., 1999). The result is a gene that is effectively inactivated and unable to produce a functional gene product. In all the mutants that I worked on, the T-DNA insert was located in the promoter region, 5′ UTR, coding region or in introns (Table 4), so I expected no transcript to be detected using primer sets that bound downstream of the T-DNA. A negative result would be

Table 7. RT-PCR primers and expected product sizes for each PP2A C subunit.

Subunit	Allele Number	Forward Primer and Reverse Primer	cDNA Product Size (bp)
C1	*c1-1*	2AC1NPU and RTPCR-C102599-L-2	115
C2	*c2-1*	RNAs150673-F and RNAs150673-R	816
C3	*c3-1*	RTPCR/C3U and 2AC3NPL	1325
	c3-2		
	c3-3		
C4	*c4-1*	RTPCRC4s035009U and RTPCR/C4s035009L	361
	c4-2		
	c4-3		
	c4-4		
	c4-5		
	c4-6		
C5	*c5-1*	RNAs013178-F and RNAs013178-R	615
	c5-2		

taaatatccattttgtgtgatatgagagaaacaaaaccaaagacgagccagagccacctctaacaccatcggcgacttctcc

C1RTPCRUU1→

tcctactcctccgtcgccgacgtgttcgatttacaacgattcggtggctattggttgtaagatttagaattaaaggttaaaggaga
*cgacgacgagag***ATG**CCGTTAAACGGAGATCTCGACCGTCAGATCGAACAGCTAAT
GGAGTGTAAGCCGTTAGGTGAAGCAGACGTGAAGATCCTTTGCGATCAAGCT
AAAGCGATTCTTGTTGAGGAATATAATGTTCAACCGGTTAAGTGTCCGGTTAC
GGTATGCGGCGATATCCATGGCCAGTTTTATGACCTAATTGAGCTATTTCGTA
TTGGTGGTAATGCTCCTGATACTAATTACCTCTTCATGGGAGATTATGTAGATC
GTGGCTACTATTCTGTAGAAACAGTCTCTCTATTGGTGGCATTAAAGGTGCGT
TACAGGGACAGACTTACGATCCTGCGAGGGAATCATGAGAGCCGTCAGATTA
CACAAGTCTATGGTTTTTATGACGAATGCTTGAGGAAATACGGAAATGCAAAT
GTGTGGAAGTATTTTACGGACCTTTTCGATTATCTCCCTCTTACAGCACTCATA
GAGAGTCAGGTTTTCTGTTTGCATGGAGGCCTTTCACCTTCTCTGGATACTCTT
GACAATATCCGAAGCTTGGATCGAATACAAGAGGTTCCACACGAAGGACCAA
TGTGCGATCTACTCTGGTCTGATCCCGACGATCGTTGTGGATGGGGAATATCT
CCTCGTGGTGCTGGTTACACGTTTGGACAGGACATTGCTACTCAGTTTAATCA
TAACAATGGACTGAGTCTGATCTCAAGAGCGCATCAACTTGTAATGGAAGGCT
ATAATTGGTGTCAGGAAAAGAACGTAGTGACAGTGTTTAGTGCACCGAACTA
CTGTTACAGATGTGGAAACATGGCCGCAATTCTTGAGATTGGAGAAAAGATG
GAACAGAACTTCCTTCAATTCGATCCAGCACCTAGACAAGTCGAACCCGATAC

c1-1

CACGCGCA▼AGACCCCTGATTATTTTTTG**TGA***tttcatttttttcttccaaagtttgtttgttgctgta*

2AC1NPU→

tcattgtagatgtgtctgttttatttgttttttcgagtctctagatggaatgtgataccaaagacgaaaaacccatcatttttttgttga

←RTPCR-C1102599-L-2

tgttgatactgaaacaggtttgtagaagcctcttcttattatagaaaatgtctttgata

Figure 12. Spliced sequence of the *Arabidopsis* PP2A *C1* gene (At1g59830). The start and stop codons are in bold. Coding sequence is in capitals. The 5′ UTR and 3′ UTR sequences are in italics. The confirmed location of the T-DNA is marked by ▼. The locations of primers are underlined, the primer name appears above, and the orientation of primers is denoted by arrows.

aaaaaaaaaaaacgaatatccatttttctcagccaggtgagagagagagacatcgaactatcgaagaccagagaagcta
c2-1
ggaggatttgatctctccgattcggatat▼*cttagctctggccgatagattctccggcgattcatcttctttatccgccgtaattttc*
*gtatataggatcggtggtggcttttcaag***ATG**CCGTCGAACGGAGATCTGGACCGTCAGATCG
AGCAGCTGATGGAGTGTAAACCGTTATCGGAGGCGGATGTGAGGACGCTTTG
CGATCAAGCGAGAGCGATCCTTGTCGAGGAATATAATGTTCAGCCGGTGAAG
TGTCCTGTTACCGTTTGCGGCGATATTCACGGCCAGTTTTATGACCTTATTGA
RNAs150673-F→
GCTCTTTCGTATCGGTGGCAACGCTCCTGATACTAACTACCTCTTCATGGGAG
ACTATGTAGATCGTGGCTACTATTCAGTAGAGACAGTTTCTCTATTGGTGGCA
CTAAAAGTGCGATACAGGGATAGACTTACAATCTTACGAGGGAATCACGAGA
GTCGGCAGATTACTCAAGTCTATGGTTTTTATGACGAATGCTTGAGGAAGTAC
GGAAATGCTAACGTCTGGAAGTATTTTACAGACCTTTTCGATTATCTTCCTCTT
ACCGCCCTCATAGAGAGTCAGGTTTTCTGTTTGCATGGAGGGCTTTCACCTTCT
CTGGATACTCTTGATAATATCCGAAGCTTGGATCGGATACAGGAGGTTCCACA
CGAAGGACCTATGTGTGATTTATTATGGTCTGATCCTGATGATCGATGTGGAT
GGGGAATATCTCCACGAGGTGCTGGTTATACATTTGGACAGGATATCGCAGCT
CAATTTAATCACAACAATGGACTAAGTCTCATATCAAGAGCGCATCAACTTGT
CATGGAAGGTTTTAACTGGTGTCAGGATAAGAATGTGGTGACTGTGTTTAGTG
CACCAAACTATTGCTACCGGTGTGGAAACATGGCTGCCATTCTAGAGATAGGA
GAGAACATGGAGCAAAACTTCCTCCAGTTCGATCCAGCTCCTCGACAAGTTGA
ACCTGATACTACTCGGAAGACCCCTGATTATTTTTTGT**TGA***tttctctgttttttttgtggtcccg*
←RNAs150673-R
accatatataatttttgaagttccagttttattgcctatgtatcattgtagatgtgccctttctcttcttttttctaagtggagttcgaatt
tcccctagaaaaaaagaaacacatcatttttttctgttgttgaggtgattgctataaaaaagtttgtaggagaggcttctatatcta
taaaattgagcgtttgtctt

Figure 13. Spliced sequence of the *Arabidopsis* PP2A *C2* gene (At1g10430). The start and stop codons are in bold. Coding sequence is in capitals. The 5′ UTR and 3′ UTR sequences are in italics. The confirmed location of the T-DNA is marked by ▼. The locations of primers are underlined, the primer name appears above, and the orientation of primers is denoted by arrows.

c3-3

gataaaaaagagagagcgagactgtgagactagagagagaga▼gctttatgaagcgaaggcaaaagtctctcgagacgattata

c3-2

taag▼ctgaaaccggttcagttaaaccgtagaagttagtattaaaccggtgatggctattgcaccaattaaagtagaggggtattatg
gtaatagcaaaagaaagagctcgaaagatacaaaccactcgcttctctcctcacgcgccttctcaaaattcgatcttctttcactgcaca
caaag*cgaagagacaaagaagagagagagagagagagagagagagagagagagagagagagagagagaaagtgggggaa*
aaaaggcgaagaatacgcagattccatccaatttagggttaggacttcccctaatttggaaacctagtcctgtttcctgatctgtttt
*ccggatcggacgagagacgaattaacgaaa***ATG**GGCGCGAATTCTATTCCGACGGACGCAACC
ATTGATCTTGATGAGCAGATCTCGCAGCTCATGCAGTGCAAGCCTCTCTCGGA

c3-1 RTPCR/C3U→

GCAACAG▼GTTAGAGCATTATGCGAGAAAGCCAAGGAGATCTTAATGGATGAA
AGCAATGTTCAGCCTGTGAAAAGCCCTGTGACAATCTGCGGTGATATTCATGGA
CAGTTCCATGATCTTGCAGAGCTTTTCCGTATAGGGGGAATGTGCCCTGATACC
AATTACCTGTTTATGGGAGACTATGTGGACCGTGGTTATTATTCTGTTGAAACTG
TTACGCTGTTAGTCGCCTTAAAGATGCGATATCCTCAGCGAATCACTATTCTTAG
AGGAAACCATGAAAGTCGTCAGATTACTCAGGTTTATGGATTTTATGATGAATG
TCTACGAAAGTACGGCAACGCAAATGTTTGGAAAATCTTTACAGACCTCTTCGA
CTATTTTCCTCTGACAGCCTTGGTTGAGTCAGAAATATTTTGCCTTCATGGTGGA
TTATCTCCATCTATCGAGACCCTTGACAACATAAGGAATTTTGATCGAGTTCAA
GAAGTGCCCCATGAAGGGCCGATGTGTGACTTATTATGGTCTGACCCCGATGAC
CGATGTGGTTGGGGCATCTCTCCTCGGGGTGCCGGATATACATTTGGTCAGGAT
ATATCTGAACAATTCAATCACACAAACAACTTAAAGCTGATCGCCCGAGCTCAC
CAGTTGGTTATGGATGGATACAACTGGGCTCACGAGCAAAAAGTGGTTACTATT
TTCAGTGCACCAAACTATTGTTACCGTTGTGGGAACATGGCCTCGATTCTTGAG
GTCGACGACTGCAGGAACCACACCTTCATCCAGTTTGAACCAGCACCGAGGAG
AGGAGAACCAGACGTCACCCGAAGGACTCCAGACTATTTCCTG**TGA***tcactggacac*
agactcatctcaatcagcagctgcaggcacccacaagtgaaaagtcacagttggctaactctagattctggtcctatgcattgtgt
tcaggttgctctgtttaaaattcaacttagtgcccaccaccggaatatagtgtgtattataggcgaagaaggcttcttatcttcctctat
aaccaagaaaagaaaaaagatacccattatgaacattgttttgttgatgttattagcttcttcttctccttctgcatcagaatgtg
gtttcatgtgagaaatcttctaattttatttcacgttattcgttcttgaacatctctgatttttgaatagaaccttgaaattgttatgtttat

←2AC3NPL

ttgttcttcaaagcttgatataataacaatagtcgctgaacgcatcagatcagccactcaaa

Figure 14. Spliced sequence of the *Arabidopsis* PP2A *C3* gene (At2g42500). The start and stop codons are in bold. Coding sequence is in capitals. The 5′ UTR and 3′ UTR sequences are in italics. The confirmed locations of T-DNA are marked by ▼. The locations of primers are underlined, the primer name appears above, and the orientation of primers is denoted by arrows.

aatttaattgtatctaacttaaaaacgattcaacttatatgtaaaaaaaagttcaaaccatatttatttccaaaaatagagtaatctaaatc

c4-5

tttcatgaacctatattttg▼aatattatttttatgttcggactctcatgaattgtcaataattagagatttcttctttttttttggttaaatttat
ttttcatgaaggagcaaatcaatttgtattttacattcatcgttcataaattttagtatgcatcattagagagaataaaaatatatatgtcac
ataaattttggtcaaaattttatgttgtaaccattcaaattttaattaaaatgatgttttggctaaatcaaaattacatttcattagctatgaat
aaatagttgataatgtattttcccatagctagacccttatgtaaacacattttacttagattatagttttttgagaaaaaactaaaatgtttgt
taaaaatgaaaaattcttcaaaacaatagttgtattgtatgtttatatagttttgtgtgtttaattaaaagaaataaaactactttacaaa

c4-3

ttttgtgtgtattacctatttaaacac▼ttttataagaaaactaaagGgtgttttagtcaatttatcatttagatctcaacgaggaggat

c4-4

ataatggtcaaa▼tcaagacacagctcgaaagctacaaacccactcgccccttacctcacgcgcccc*tttgttttgatttcctct*

c4-6

ctcactctgcacacgaaggcgagagagagagagaaagaaa▼aaagcgaagaatacgcagaatccatccaatttaggg

RTPCRC4-2S-F-2→

ttagggctgcccctaatctggaaacctttgcctcgtttctgatctgttttccggatccgacttgaaggaaagaagacgacgacg
*cactaccaaaaac*ATGGGCGCGAATTCGCTTCCAACGGATGCAACCCTCGATCTAGA

c4-2

TGAGCAGATCTCGCAGCTCATGCAGTGCAAGCCTCTCTCCGAGCAACAG▼GT
CAGAGCATTATGTGAGAAAGCTAAGGAGATCTTAATGGATGAAAGCAACGTT
CAGCCTGTTAAAAGCCCTGTGACAATCTGCGGTGATATTCATGGACAGTTCCA
TGATCTTGCAGAGCTTTTCCGTATTGGGGGAAAGTGTCCTGATACCAACTATC
TGTTTATGGGAGACTATGTCGACCGTGGATATTATTCTGTGGAAACTGTTACG
CTGTTGGTTGGCTTGAAAGTACGGTATCCACAGCGAATCACTATTCTTAGAGG
AAACCATGAAAGTCGTCAGATTACTCAGGTTTATGGATTTTATGATGAATGTC
TGCGAAAGTATGGCAATGCAAATGTTTGGAAGATATTTACAGACCTCTTTGAC
TATTTCCCACTGACAGCCTTGGTGGAGTCGGAAATATTCTGCCTTCACGGTGG
ATTATCACCATCCATCGAGACCCTTGACAACATTAGGAACTTTGATCGGGTTC
AAGAAGTTCCACATGAAGGGCCTATGTGTGACTTATTATGGTCTGATCCTGAT
GACAGATGTGGCTGGGGAATCTCTCCTCGTGGTGCTGGATATACATTTGGTCA
GGATATTTCGGAACAATTCAATCACACTAACAACTTAAAGCTGATCGCCCGAG

c4-1

C▼GCATCAGCTCGTTATGGATGGATTCAATTGGGCACACGAGCAAAAAGTG

RTPCR/C4s035009U→

GTTACTATATTCAGTGCACCAAACTATTGCTATCGCTGTGGAAACATGGCCTC
AATTCTTGAGGTCGATGACTGCAGGAACCACACGTTCATTCAGTTTGAACCAG
CACCGAGGAGAGGAGAGCCAGATGTAACCCGAAGGACACCTGACTATTTCCT
TTGA*agatactcagctcctcctgcagcttccggttgttggtttttgaagatctctgcttattccatttggttcaacgcatttgtgttc*
caggttgcttctctttaaaatcaatttagccctctcccttggaatagtgtaatataggcaaagaggtcaatgaactttaaaacctct

←RTPCRC4-2S-R-2 ←RTPCR/C4s035009L

catgaacatgtgtttggctgaacttattagctcccttctgcatcagaatctgtttattgtgagatatttttttcgatttatttcacgtaatttt
tccctttgttccatatatctgtttcgtgcacctttttatttgaaacctaatatataaatcgaaatgctgttctttcaagcc

Figure 15. Spliced sequence of the *Arabidopsis* PP2A *C4* gene (At3g58500). The start and stop codons are in bold. Coding sequence is in capitals. The 5′ UTR and 3′ UTR sequences are in italics. The confirmed locations of the T-DNA are marked by ▼. The locations of primers are underlined, the primer name appears above, and the orientation of primers is denoted by arrows.

c5-1

aatgatgattagggtttactaatttggaattaggagttacatttaaggttaaaagtttatgagtttcaagtttcaac▼gaagctttaaag
tttaaacgatggttaaagttttatgattatggtgggctatgttatagttctttcaatttcgttgggccaaatgggctaaacgattaacgac
cctatgaatgggcctgaaaaaatgaaagccacgtgtcagacccccacaatggactttgactcggtgcggctgccggagagaat*a*
attaattatcaactccgatgaaaagaaaacaaatacaaagttccaacaaaaatcgaaattcacaatttctcttcttctaaatcc
atggagtgatgatgatgatagatagattgaatccaaacacaaaccggatccgagattaaaaacaatccggattgtttatattta
*ctttagatcggagaaag***ATG**CCGCCGGCGACCGGAGATATCGATCGTCAGATCGAGCA
GCTTATGGAGTGTAAAGCGTTATCTGAAACGGAGGTGAAGATGTTGTGTGAG
CACGCAAAGACGATTCTTGTGGAAGAGTATAATGTTCAACCGGTTAAATGTCC
GGTTACCGTCTGCGGTGATATCCACGGCCAATTTTACGATCTAATCGAGCTT

2AC513→

TTTCGTATCGGTGGTTCTTCTCCTGATACTAATTATCTTTTCATGGGTGATTAT

c5-2

GTTG▼ATCGAGGGTATTATTCTGTGGAGACAGTCTCACTTTTGGTAGCACTGA
AAGTTCGTTACAGAGATAGACTTACTATCCTAAGAGGGAATCATGAAAGCC

RNAs013178-F→

GTCAAATTACTCAAGTGTATGGGTTTTATGATGAATGTTTGAGGAAATATGGA
AATGCTAATGTATGGAAGCACTTCACTGATCTTTTTGATTATCTTCCACTTACA
GCTCTTATTGAGAGTCAGGTTTTCTGTTTACATGGAGGACTTTCACCTTCTTTA
GATACACTTGACAACATCCGATCTTTAGATCGAATTCAAGAGGTTCCACATGA
AGGACCAATGTGTGATCTCTTATGGTCTGATCCAGATGACCGATGCGGTTGGG
GAATATCTCCTCGTGGTGCAGGCTACACTTTCGGACAAGATATCGCTACTAGT
TTAACCACACCAATGGACTCTCTCTGATTTCAAGAGCACATCAACTTGTCATG
GAAGGTTTTAATTGGTGCCAAGAAAAGAACGTTGTGACTGTATTTAGTGCCCC
AAACTATTGCTACCGTTGTGGCAACATGGCTGCGATTCTAGAGATCGGTGAGA
ACATGGACCAGAATTTCCTTCAGTTTGATCCAGCTCCACGTCAAGTCGAACCC
GAAACCACTCGCAAGACTCCAGATTATTTTTTGT**TAA***gtaccaaaagaactaaagctgcaatt*

←RNAs013178-R

ccacacactagctcttcgtctctttgcttctattcttaaccacttttgtaatttcatattccttacgtctatttctgtttgttaaactctcca
ttgtcttttaaaatccgtaatttttgtgtgcaaagattctggtccaagtaactgaaaccgctatcacc

Figure 16. Spliced sequence of the *Arabidopsis* PP2A *C5* gene (At1g69960). The start and stop codons are in bold. Coding sequence is in capitals. The 5′ UTR and 3′ UTR sequences are in italics. The confirmed locations of the T-DNA are marked by ▼. The locations of primers are underlined, the primer name appears above, and the orientation of primers is denoted by arrows.

inconclusive since a failed reaction could also be responsible for no RT-PCR product. Therefore, several control reactions were performed simultaneously. The first control tested the ability of the RNA to function as a template in RT-PCR using actin gene primers. The second control tested the amplification ability of the C subunit primers using wild type RNA as template. The third control tested the ability of the C subunit primers to amplify a product from genomic DNA of the *C* gene mutants, ruling out the possibility that the primer binding sites may have undergone a point mutation or some other type of alteration.

In alleles *c3-1, c3-3* and *c4-1*, mRNA downstream of the T-DNA insertion was absent (Table 8). The first two control reactions showed that both RNA and primers were functional for all three mutants. The third control proved that the primers were capable of amplifying from mutant genomic DNA in allele *c4-1*. However, in alleles *c3-1* and *c3-3*, the RT-PCR primers were designed to bind to cDNA but not genomic DNA, so PCR could not be conducted with these primers. Experiments with new primers are in progress. These results indicated that the T-DNAs resulted in a knockout in alleles *c3-1, c3-3* and *c4-1*. An example of an RT-PCR experiment is shown in Figure 17.

In the other 10 alleles, a transcript was detected using primers located downstream of the T-DNA (Table 8). The most likely explanation for these results is that the amplified product is from a partial transcript initiated from a promoter within the T-DNA. In cases where the T-DNA has inserted in the promoter region or 5′ UTR, a downstream transcript would contain full-length coding sequence (Table 8). Seven of the C subunit mutants fall into this category: *c2-1, c3-2, c3-3, c4-3, c4-4, c4-5, c4-6* and *c5-1*.

Table 8. Transcript detection in PP2A T-DNA insertion lines by RT-PCR using primers downstream of the T-DNA.

PP2A Subunit	Allele Number	T-DNA Location	Transcript Detected
C1	*c1-1*	6th of 6 exons	Yes
C2	*c2-1*	5′ UTR	Yes
C3	*c3-1*	1st of 10 introns	No
	c3-2	Promoter	Yes
	c3-3	Promoter	No
C4	*c4-1*	9th of 11 exons	No
	c4-2	1st of 10 introns	Yes
	c4-3	Promoter	Yes
	c4-4	Promoter	Yes
	c4-5	Promoter	Yes
	c4-6	5′ UTR	Yes
C5	*c5-1*	Promoter	Yes
	c5-2	1st of 4 introns	Yes

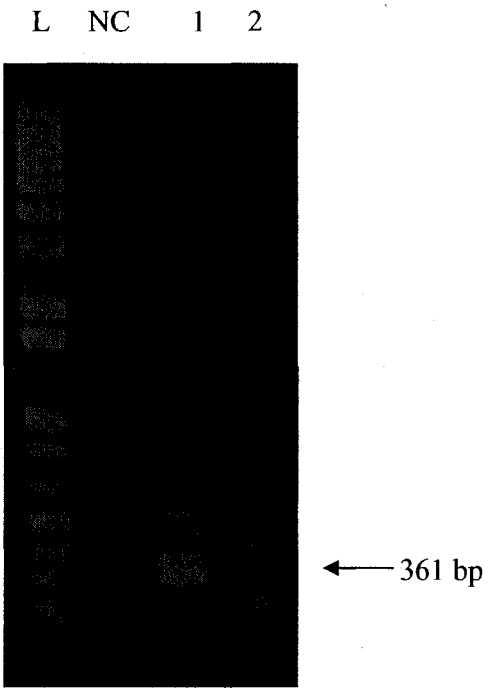


Figure 17. RT-PCR to detect transcript from the PP2A *c4-1* mutant using primers located downstream of the T-DNA insertion. Reaction products were separated by electrophoresis, stained with ethidium bromide and visualized under UV light. Expected cDNA product size = 361 bp. Lane L: size marker. Lane NC: negative control (no RNA). Lane 1: RNA template from wild type plants. Lane 2: RNA template from homozygous *c4-1* mutant plants. A second RT-PCR positive control using actin gene primers on homozygous c4-1 mutant plants showed that the RNA template from homozygous *c4-1* mutant plants was functional (data not shown).

For the remaining three C subunit mutant lines (alleles *c1-1*, *c4-2* and *c5-2*) for which downstream transcript was detected, the T-DNA had inserted in an exon or intron. Even though a partial transcript initiated from a promoter within the T-DNA was detected, I hypothesized that no full-length transcript would be produced. To detect the presence of full-length transcript in these three alleles, additional RT-PCR was performed using one primer located before and the other primer after the T-DNA insertion site. The observed and expected RT-PCR products for these reactions are shown in Table 9. Primers are marked on the C subunit gene sequences in Figures 12, 15 and 16. For alleles *c1-1* and *c4-2*, no mRNA was detected with these flanking primer sets indicating that no full-length transcript was produced (Table 9). For allele *c5-2*, mRNA was detected with the flanking primer set. One possible explanation for the presence of transcript for the *c5-2* allele is that, since the T-DNA is located in an intron, the T-DNA may be spliced out allowing the native transcript to be produced.

In summary, five mutants have been confirmed to lack a full-length transcript: *c1-1*, *c3-1*, *c3-3*, *c4-1* and *c4-2*. These represent the gene knockouts that I was looking for. Next, phenotype screening was done with these knockout lines.

Phenotype Screening

Since a knockout plant does not produce a functional gene product, such plants are the best candidates to screen for potential phenotypes. Initially, plants containing the *c1-1*, *c3-1*, *c3-3* and *c4-1* alleles were used to conduct phenotype screenings since RT-PCR revealed that these mutants were unable to produce full-length mRNA. I did not use the *c4-2* mutant because it was not available when I started phenotype screenings. These

Table 9. Transcript detection in PP2A T-DNA insertion lines by RT-PCR using primers flanking the T-DNA.

Allele Number	T-DNA Insertion Site	Primers Flanking T-DNA	cDNA Product Size (bp)	Transcript Detected
c1-1	6th of 6 exons	C1RTPCRUU1 and RTPCR-C1102599-L	1130	No
c4-2	1st of 10 introns	RTPCRC4-2S-F-2 and RTPCRC4-2S-R-2	1182	No
c5-2	1st of 4 introns	2AC513 and RNAs013178-R	779	Yes

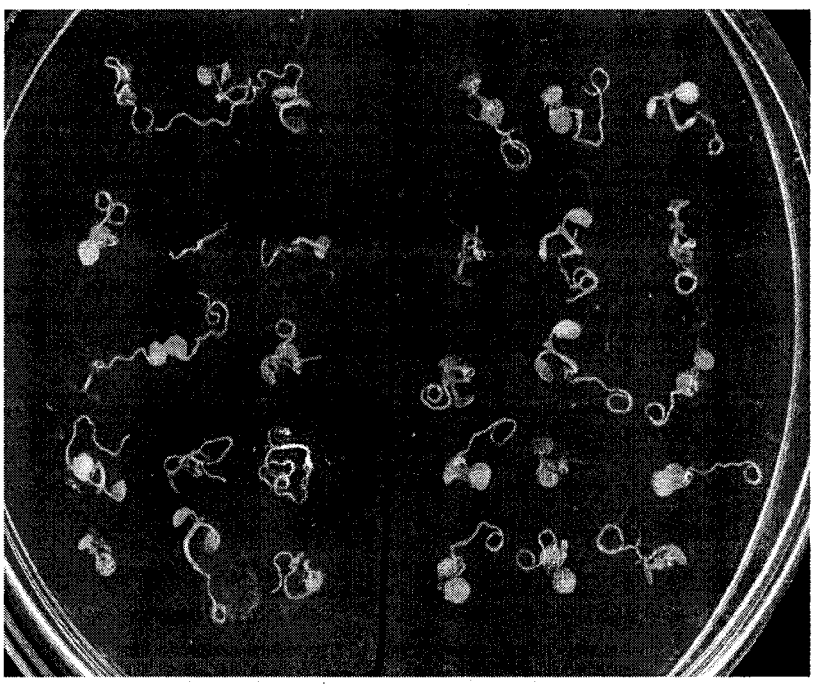
mutant plants were first grown on plates and transplanted to soil under normal growth conditions. No obvious phenotype was identified when they were compared to wild type plants. Then I started screening for phenotypes under a variety of growth conditions and treatments.

Root Curling Assay

This assay tested whether plant roots from the PP2A mutants had altered root curling as compared to wild type roots. Fifteen wild type seeds and fifteen PP2A mutant seeds were sown on a plate containing Low Sucrose MS medium. The roots from the *c1-1*, *c3-1*, *c3-3*, and *c4-1* mutant seedlings coiled into loops similar to the wild type roots. An example comparing wild type and *c4-1* mutant seedlings is shown in Figure 18.

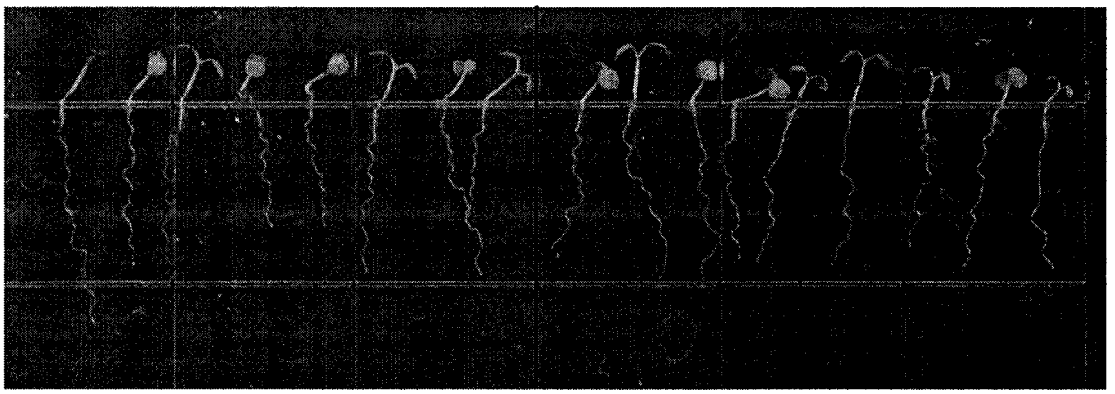
Root Waving Assay

To compare the root waving characteristics of wild type and mutant plants, seeds were sown in a line on a plate made with Low Sucrose MS medium containing 1.5% agar. Eight sterilized wild type seeds were placed on the left side of each plate and eight seeds of a PP2A mutant were placed on the right side. Two plates were used for each mutant line. The roots from *c1-1*, *c3-1*, *c3-3*, and *c4-1* mutant seedlings adopted a wavy pattern of growth similar to the wild type plants. There was no visual difference between wild type and PP2A mutant plants. An example comparing wild type and *c4-1* mutant seedlings is shown in Figure 19.



WT *c4-1*

Figure 18. Root curling assay on wild type (WT) and PP2A *c4-1* mutant seedlings.



WT *c4-1*

Figure 19. Root waving response of wild type (WT) and PP2A *c4-1* mutant seedlings.

Germination Assay

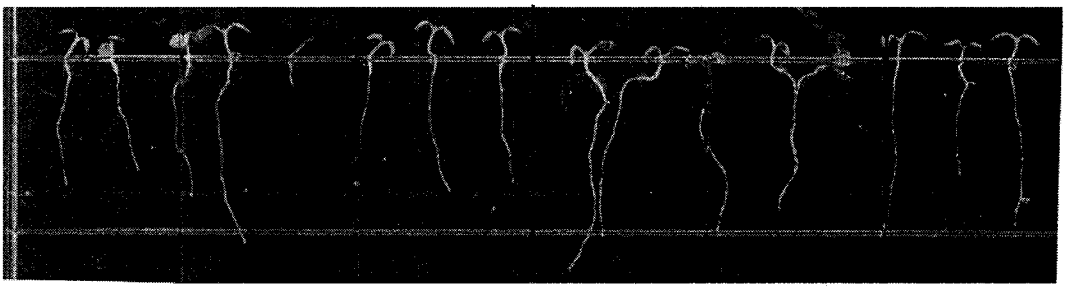
To test germination of mutant and wild type plants, nine sterilized wild type seeds were placed on the left side of each plate and nine seeds of PP2A mutants were placed on the right side. Four plates were used for each mutant line. Seeds were sown on a plate containing Low Sucrose MS medium. After three days in standard growth conditions, seeds were evaluated for germination by observing the presence of a radicle. Germination of the wild type plants ranged from 94% to 100%. Germination of the *c1-1*, *c3-1*, *c3-3*, and *c4-1* mutant plants reached 100%. There was no significant difference in germination between wild type and PP2A mutant plants ($p>0.7$; Table 10). Thus, germination of wild type and PP2A mutant plants was similar.

Growth on Vertically-oriented Plates

To test the gravity responsiveness of PP2A mutants and wild type plants, eight seeds of each genotype were sown on plates containing Low Sucrose MS medium made with half-strength MS. Two plates were used for each mutant line. Plates were oriented vertically. After growing in standard conditions for 5 days, root growth and direction was visually compared between wild type and mutant plants. The roots from PP2A *c1-1*, *c3-1*, *c3-3*, and *c4-1* mutant seedlings grew downward along the gravity vector and so did all wild type seedlings. There was no obvious difference between the appearance of the PP2A mutant and wild type seedlings in vertically-oriented plates. An example comparing wild type and *c4-1* mutant seedlings is shown in Figure 20.

Table 10. Germination of PP2A mutant and wild type seeds.

PP2A Subunit Gene	Allele	Wild Type Germination	Mutant Germination
C1	*c1-1*	94%	100%
C3	*c3-1*	100%	100%
	c3-3	100%	100%
C4	*c4-1*	94%	100%



WT *c4-1*

Figure 20. Growth of wild type (WT) and PP2A *c4-1* mutant seedlings on vertically-oriented plates.

Growth on Vertically-oriented Plates Followed by 90° Rotation

An experiment was conducted to test the gravity responsiveness of PP2A mutant and wild type seedlings. Six seeds of each genotype were sown on plates containing Low Sucrose MS medium made with half-strength MS. Two plates were used for each mutant line. After growth for four days on a vertically-oriented plate containing Low Sucrose MS medium made with half-strength MS, the roots of both PP2A mutant and wild type seedlings grew downward due to gravity (data not shown). The plates were then rotated counterclockwise 90° in the vertical plane on the fourth day. The roots of both PP2A mutant and wild type seedlings re-oriented and started growing downward due to gravity. After four days, the angle of reorientation appeared to be ~90° in all of the PP2A *c1-1*, *c3-1*, *c3-3*, and *c4-1* mutants as well as the wild type plants. There was no difference between PP2A mutant and wild type plants following vertical re-orientation. An example comparing wild type and *c4-1* mutant seedlings is shown in Figure 21.

Growth on Medium Containing Ethanol

To test the response of mutant and wild type plants to growth in the presence of ethanol, nine seeds of each genotype were sown on plates containing Low Sucrose MS medium plus 0.5% (v/v) ethanol. Four plates were used for each mutant line. In each plate one row of wild type seeds was arranged on the left side of the plate and mutant seeds were on the right side of the plate. After incubation under standard growth conditions for 6 days, seeds were scored as germinated if a radicle was present. None of the wild type or mutant seeds germinated.

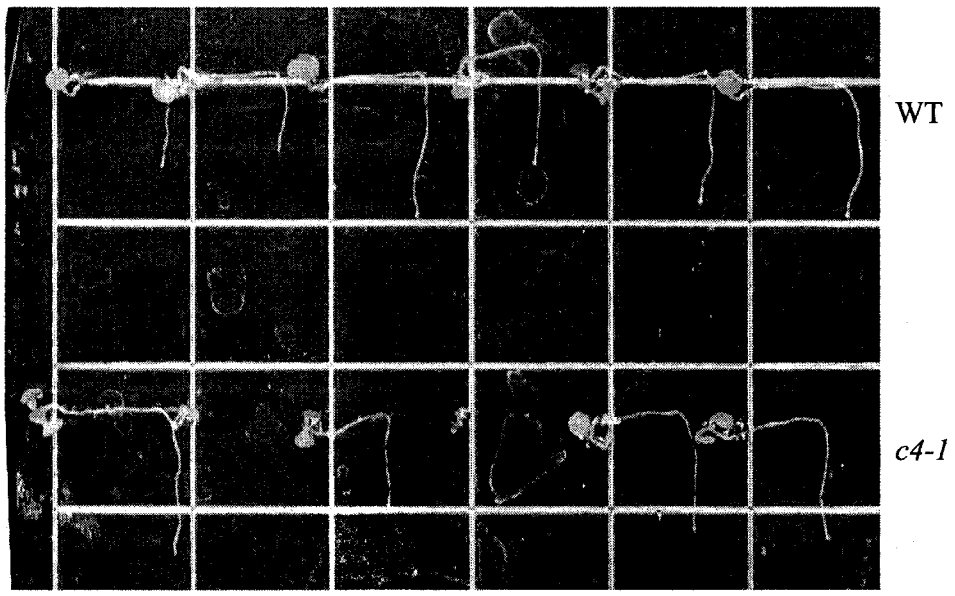


Figure 21. Growth of wild type (WT) and PP2A *c4-1* mutant seedlings on vertically re-oriented plates.

Growth on Medium with Additional Sucrose

To compare the sucrose response of PP2A mutant and wild type plants, six seeds of each genotype were sown on a plates containing half strength MS plus 2% sucrose. Two plates were used for each genotype. Six sterilized wild type seeds were plated on the left side of each plate and six seeds of a PP2A mutant were plated on the right side. After 5 days in standard conditions, the growth of roots was compared between PP2A mutant and wild type seedlings. The roots from wild type plants and *c1-1*, *c3-1*, *c3-3*, and *c4-1* mutant plants grew along the gravity vector. There was no visible difference in root length between PP2A mutant and wild type seedlings in response to additional sucrose. An example comparing wild type and *c4-1* mutant seedlings is shown in Figure 22.

Growth at 15°C

The effect of low growth temperature was tested for PP2A mutants and wild type seedlings. The plates contained Low Sucrose MS medium. Eight sterilized wild type seeds were plated on the left side of each plate and eight seeds of a PP2A mutant were plated on the right side. Two plates were used for each genotype. The plates were placed in a growth chamber with a 12 hour photoperiod and a constant 15°C temperature. All of the PP2A seeds from the *c1-1*, *c3-1*, *c3-3*, and *c4-1* mutants germinated and most of the wild type seeds germinated. The roots from both the PP2A mutants and wild type plants grew along the gravity vector. There was no visible difference in root length between PP2A mutant and wild type seedlings in response to growth at 15°C. All plants maintained a healthy green color but grew at a very slow rate. An example comparing wild type and *c4-1* mutant seedlings is shown in Figure 23.

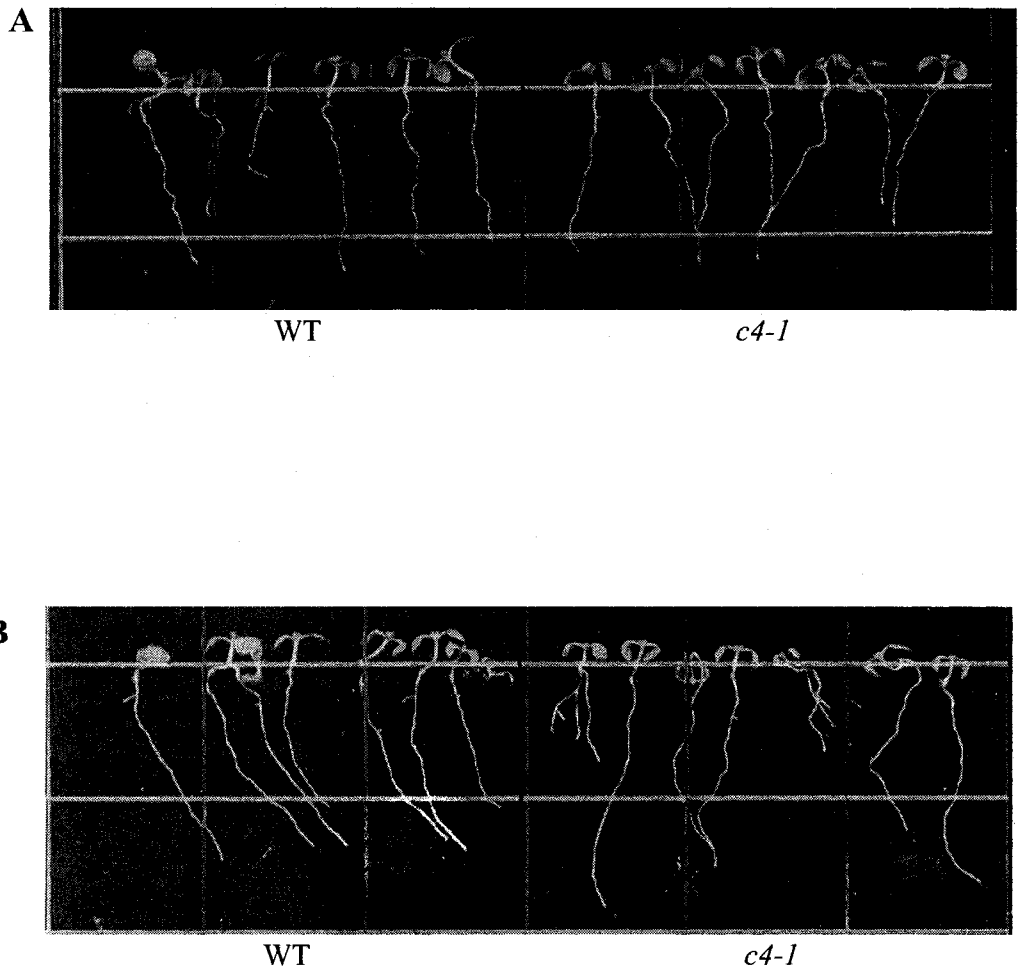
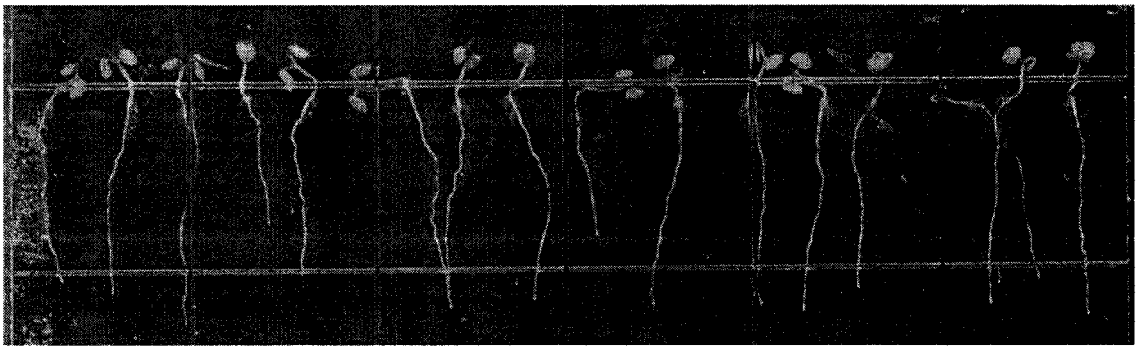


Figure 22. Root growth of wild type (WT) and PP2A *c4-1* mutant plants on medium with 2% sucrose. **A.** ½ MS + 2% (w/v) sucrose. **B.** ½ MS only.



WT *c4-1*

Figure 23. Growth of wild type (WT) and PP2A *c4-1* mutant seedlings at 15°C.

Growth on Medium Containing ACC

Ethylene inhibits hypocotyl elongation of the wild type plants grown in the dark (Johnson and Ecker, 1998). To examine the ethylene response, seven seeds of PP2A mutants and seven seeds of wild type were sown on MS medium containing no sucrose plus 25 μM ACC. Two plates were used for each genotype. The plates were exposed to light for one day then transferred to the dark. After growing in the dark for four days, hypocotyl length was compared between wild type and mutants. In the absence of ACC, hypocotyls were elongated in both the wild type and PP2A *c1-1*, *c3-1*, *c3-3*, and *c4-1* mutants (Figure 24A). In the presence of ACC, hypocotyl growth was inhibited both in the wild type and PP2A mutants. There was no visual difference in hypocotyl length between wild type and mutants in either treatment. An example comparing wild type and *c4-1* mutant seedlings is shown in Figure 24.

Growth on Medium Containing ABA

PP2A mutants were tested for ABA sensitivity by comparing germination in the presence of ABA. ABA is known to affect germination and wild type seeds grown in the presence of ABA exhibit a decrease in germination (Leung and Giraudat, 1998; Finkelstein et al., 2002). Fifteen wild type seeds and fifteen PP2A mutant seeds were sown on half-strength MS medium with no sucrose containing 1 μM ABA. Two plates were used for each genotype. After growth under standard conditions for 3 days, seeds were scored as germinated by the presence of a radicle. Germination in the absence of ABA reached 100% in the PP2A *c1-1*, *c3-1*, *c3-3*, and *c4-1* mutants and ranged from 96% to 100% in the wild type (Table 11). Germination in the presence of ABA was 10%-

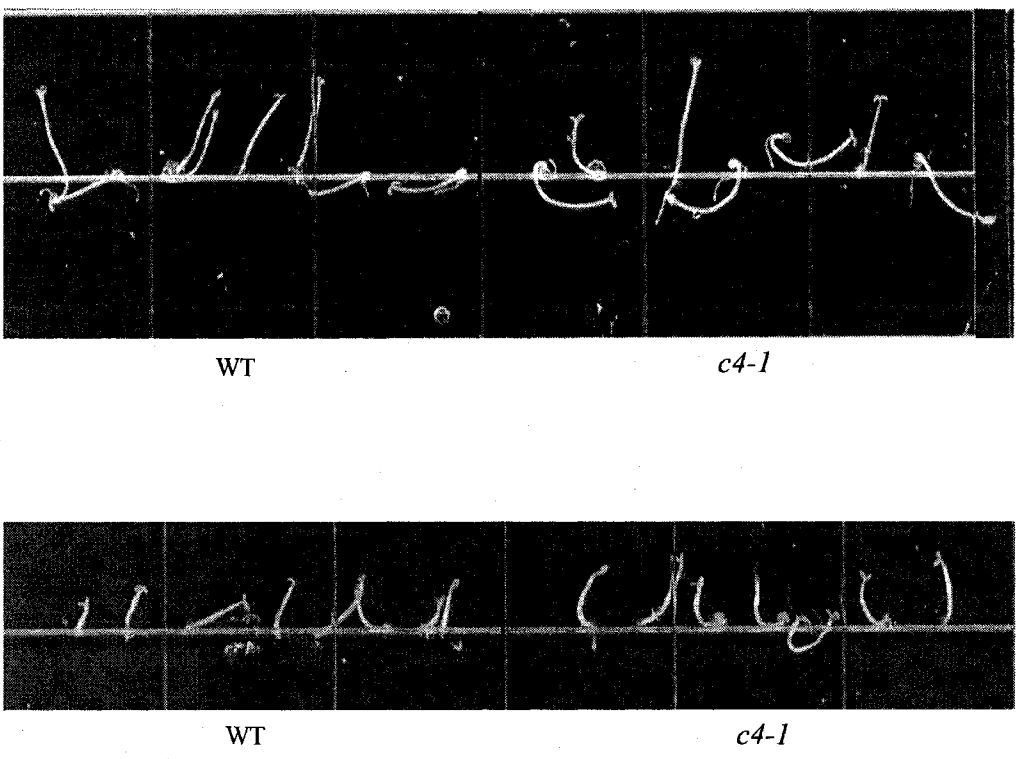


Figure 24. Hypocotyl length in the presence of ACC. Wild type (WT) and PP2A *c4-1* mutant seedlings were grown on ½ MSNS medium. **A.** No ACC. **B.** 25 μM ACC.

Table 11. Germination of PP2A mutant and wild type seeds on ABA.

Mutant Allele	Medium without ABA		Medium with 1 μM ABA	
	Wild Type	Mutant	Wild Type	Mutant
c1-1	96%	100%	10%	13%
c3-1	100%	100%	13%	13%
c3-3	100%	100%	10%	13%
c4-1	100%	100%	13%	10%

13% in PP2A *c1-1*, *c3-1*, *c3-3*, and *c4-1* mutants and wild type. There was no significant difference in ABA response between wild type and PP2A mutant plants ($p>0.5$; Table 11).

DISCUSSION

To determine whether the expression of the PP2A C subunit genes identified in Chapter II is affected by the T-DNA insertion, I used an RT-PCR approach to detect transcript of PP2A C subunit genes in mutants. When T-DNA inserts in the promoter region, 5′ UTR, coding region or introns, it has been predicted that a knockout should be produced in which no downstream message or a perhaps truncated message is formed (Krysan et. al., 1999). Therefore in my study, the primary criterion for selecting alleles from the T-DNA knockout collections was that the insertion be located in the promoter region, 5′ UTR or close to the start codon. It was not possible to meet these criteria for the C1 subunit since only one allele was available and it was in the last exon. I did not select alleles with T-DNA insertions in the 3′ UTR because it is generally thought that insertions further upstream in the gene are more likely to cause complete loss-of-function (Ostergaard and Yanofsky, 2004). I used primers located after the T-DNA insertion site to detect transcript in mutant plants and I expected no downstream transcript to be detected. Among the 13 C subunit alleles investigated, no downstream mRNA was detected in the three plants *c3-1*, *c3-3* and *c4-1*. These three plants were considered to be knockout mutants. For the other alleles, downstream transcripts of the correct size were detected. To understand why expression was still occurring in many of the mutants, I checked the vector map of the T-DNA construct used in the mutants. The vector map of the T-DNA construct used by the Salk Institute showed that the T-DNA contains a 35S promoter sequence. The 35S promoter is located about 1 Kb from the left border of the T-

DNA construct (http://signal.salk.edu/tdna_protocols.html; Figure 25) facing toward the left border. A 35S promoter sequence is also present in the T-DNA construct from GABI (Figure 25), but facing toward the right border. In both cases, there is a transcription terminator sequence, but no coding sequence, downstream of the 35S promoter. Since T-DNA can insert in either orientation in relation to the genomic sequence (Figure 26), if the orientation of T-DNA is such that the 35S promoter is the same as the orientation of the gene in which it has inserted, the 35S promoter may cause downstream expression. The orientation of the 35S promoter in each of the PP2A C subunit alleles is listed in Table 12.

Since the consequences of expression from the 35S promoter differ depending on the T-DNA insertion site, I will discuss the mutants in two groups: those with T-DNA in the promoter or 5′ UTR and those with T-DNA in an exon or intron. In cases where the T-DNA has inserted in the promoter region or 5′ UTR, a downstream transcript would contain full-length coding sequence. In all eight C subunit mutant lines where T-DNA is inserted in the promoter region or 5′ UTR (alleles *c2-1, c3-2, c3-3, c4-3, c4-4, c4-5, c4-6* and *c5-1*), the orientation of the 35S promoter is the same as the orientation of the C subunit gene and a full-length transcript was detected in seven of these plants by RT-PCR (Table 8). These results indicate that a 35S promoter with the same orientation as the gene may cause expression. However, there is no experimental data in this study to prove that the 35S promoter within the T-DNA is the source of the *C* gene expression. For the eighth mutant line (*c3-3*), no gene product was detected downstream of the T-DNA even

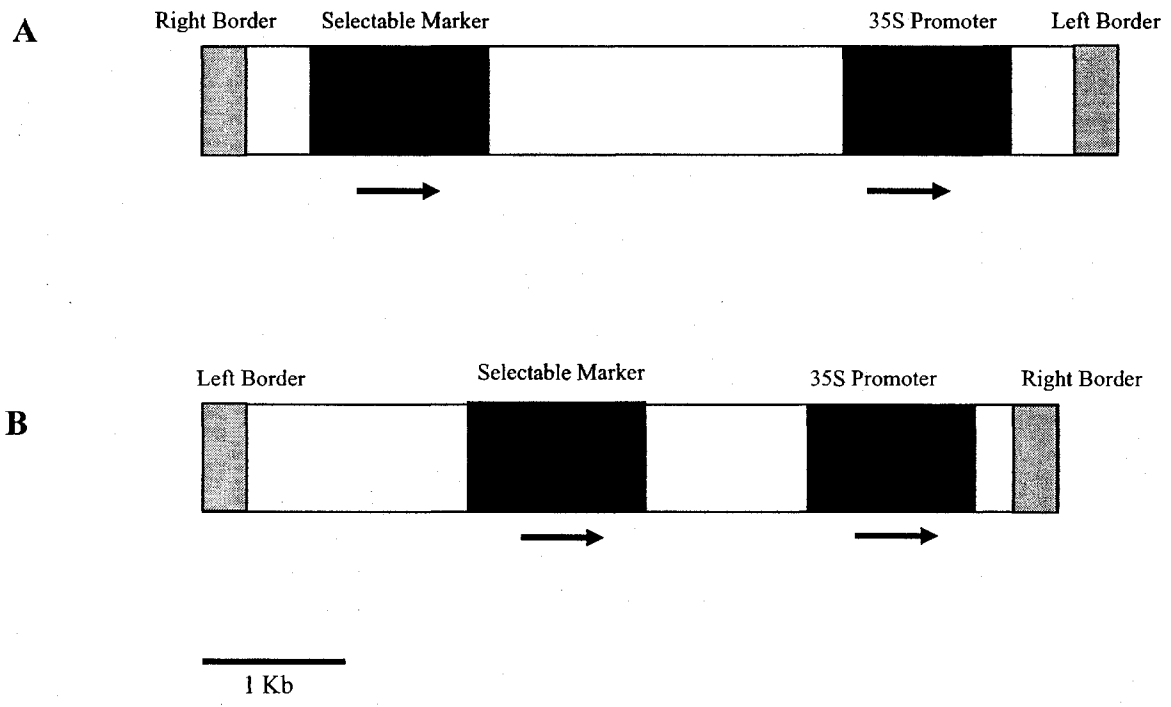


Figure 25. Schematic of the most pertinent features of the T-DNA constructs used in this study. **A.** T-DNA from Salk Institute, La Jolla, CA **B.** T-DNA from GABI, Bielefeld, Germany.

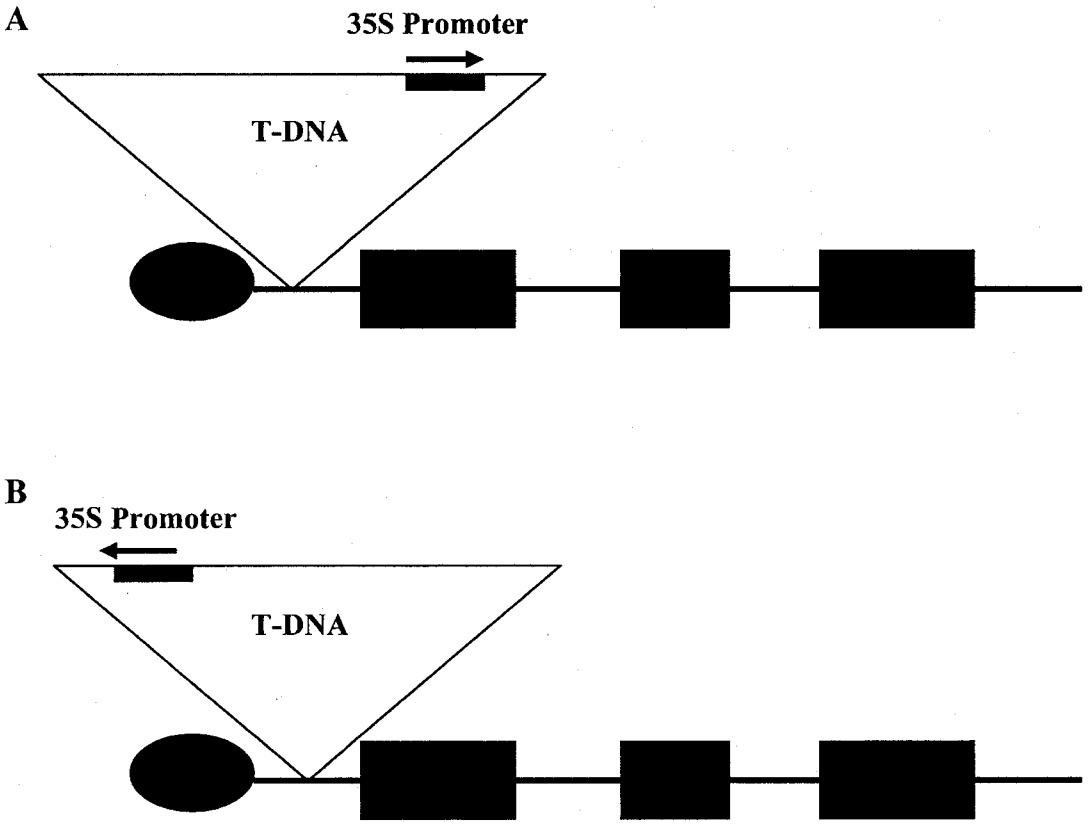


Figure 26. Schematic of the two possible orientations for T-DNA to insert in a hypothetical gene. In the hypothetical gene, a black oval represents the promoter; black rectangle is coding sequence and black line represents 5′ UTR, 3′ UTR or intron.

Table 12. Orientation of the 35S promoter in PP2A mutant lines. "→" indicates that the orientation of the 35S promoter and gene are same (see Figure 26A). "←" indicates that the orientation of the 35S promoter is opposite of the orientation of the gene (see Figure 26B).

PP2A Subunit	Allele Number	T-DNA Insertion Site	Transcript Detected	Orientation	Putative Knockout
C1	*c1-1*	6th of 6 exons	No	→	Yes
C2	*c2-1*	5′ UTR	Yes	→	No
C3	*c3-1*	1st of 10 introns	No	←	Yes
	c3-2	Promoter	Yes	→	No
	c3-3	Promoter	No	→	Yes
C4	*c4-1*	9th of 11 exons	No	←	Yes
	c4-2	1st of 10 introns	No	→	Yes
	c4-3	Promoter	Yes	→	No
	c4-4	Promoter	Yes	→	No
	c4-5	Promoter	Yes	→	No
	c4-6	5′ UTR	Yes	→	No
C5	*c5-1*	Promoter	Yes	→	No
	c5-2	1st of 4 introns	Yes	→	No

though the promoter and gene were in the same orientation. It is possible in this case that transcription does initiate but terminates before the primer site used for reverse transcription is reached or that the transcript is unstable and rapidly degraded. I concluded that, for this group of eight alleles, the T-DNA has only interrupted PP2A gene expression in *c3-3*.

In the remaining three C subunit mutant lines in which downstream transcript was detected (alleles *c1-1*, *c4-2* and *c5-2*), the T-DNA had inserted in an exon or intron. Even though a partial transcript initiated from a promoter within the T-DNA was detected, it is probable that no full-length transcript can be produced. To detect the presence of full-length transcript from these three alleles, RT-PCR was performed using a primer set flanking the T-DNA insertion. For alleles *c1-1* and *c4-2*, no mRNA was detected with these flanking primer sets indicating that the T-DNA interrupts gene expression. For allele *c5-2*, mRNA was detected with the flanking primer set. Since the T-DNA is located in an intron, the T-DNA may be spliced out allowing the native transcript to be produced. I concluded that, for this group of these alleles, *c1-1* and *c4-2* are knockouts.

Protein phosphorylation is a reversible process that affects many biological processes. Biochemical evidence has shown the functional diversity of PP2A in plants (Siegl et al., 1990; Huber et al., 1992; Li et al., 1994; MacKintosh et al., 1994; Smith et al., 1994; Monroy and Dhindsa, 1995; Foissner et al., 2002). So far, only a few PP2A mutant plants with phenotypes have been identified; these include mutations in the *Arabidopsis* A1, B″ζ and C2 subunit gene (Garbers et al., 1996; Camilleri et al., 2002; Kwak et al., 2002; Larsen and Cancel, 2003; Pernas et al., 2007). However, I believe that

there are potentially more phenotypes to be identified with PP2A mutant plants. In this research, after having isolated plant lines homozygous for T-DNA insertions in PP2A C subunit genes, the next step was to determine if the mutations resulted in a phenotype that could be distinguished from wild type. I grew four of the five PP2A homozygous single knockout mutant lines (alleles *c1-1, c3-1, c3-3 and c4-1*) in a variety of growth conditions and treatments. The protocols were obtained and modified from the *Arabidopsis* Gantlet Project website (http://thale.biol.wwu.edu/) and from the literature. The phenotype screens which I chose were done with the equipment available in the Hrabak lab.

Among the screens described in this chapter, none showed a phenotype distinguishable from wild type. The one phenotype identified for the *c4* mutants will be described in Chapter IV. Although use of a protein phosphatase inhibitor has provided significant knowledge of PP2A functions in plants (Huber et al., 1992; Li et al., 1994; MacKintosh et al., 1994; Monroy and Dhindsa, 1995; Foissner et al., 2002), none of the associated phenotypes was identified in my screens. One possible explanation is that, since a PP2A inhibitor affects all PP2A enzymes in the cell, the phenotypes identified with a pharmacological approach are likely to be due to global inhibition of PP2A activity, and possibly also PP4 activity, rather than the effect of inactivating a single subunit. Furthermore, there are five genes encoding PP2A C subunits and an amino acid alignment of these subunits reveals strong similarities (Figures 1 and 27). Thus, loss of one C subunit might be rescued by another related member of the C subunit family.

	C1	C2	C3	C4	C5
C1		93.1	77.1	76.7	89.1
C2	7.2		78.1	78.4	93.5
C3	27.4	25.9		98.1	78.5
C4	27.9	25.5	1.9		78.8
C5	11.1	6.9	25.4	24.9	

Figure 27. Pairwise distance matrix of PP2A C subunits. Values are based on the Clustal W alignment shown in Figure 1. Percent similarity is shown at upper right and percent divergence is at lower left. C1 = At1g59830, C2 = At1g10430, C3 = At2g42500, C4 = At3g58500, C5 = At1g69960.

Protein phosphatases are present in all eukaryotes and involved in most activities in cells. It may be necessary to construct higher-order knockouts to identify mutant phenotypes as has been observed with the PP2A A subunit mutants in *Arabidopsis*. Both single *a2* and *a3* mutants show no obvious phenotypes compared to wild type while double mutants between the *a1* (*rcn1*) subunit mutant and either the *a2* or *a3* mutants have much more severe morphological defects (Zhou et al., 2004). Identifying a possible phenotype also depends on choosing the correct assay conditions which is often a matter of guesswork. Mutations in genes that are involved in stress responses may only display a detectable phenotype when subjected to certain environmental challenges. It is possible that additional phenotypes of PP2A C subunit mutants might be identified if additional assay conditions were tested.

CHAPTER IV

CHARACTERIZATION OF THE ROOT SKEWING PHENOTYPE OF A PROTEIN PHOSPHATASE 2A C SUBUNIT MUTANT

Introduction

One way that plants sense and respond to environmental signals is reorientation of the direction of root growth. One example of this reorientation is termed "skewing" or "slanting". Skewing is defined as deviation of growth from the direction of gravity. This can be observed with *Arabidopsis* seedlings growing on vertical agar plates which tend to deviate from strict downward growth direction by veering slightly to one side (Simmons et al., 1995). Authors define the direction of skewing (left or right) by indicating whether plants were viewed through the lid of the plates or from behind. Different *Arabidopsis* ecotypes have different but consistent skewing angles when grown on vertically-oriented plates. Wild type Columbia (Col) seedling roots grow almost straight down (~6° left skew) while wild type Wassilewekija (WS) and Landsberg (Ler) roots always skew to the left by ~20° when viewed from the top of the plate (Rutherford and Masson, 1996).

The mechanism of plant root skewing is not fully understood but is likely induced by both touch stimuli and gravity responses. Columella cells within the root cap are believed to be the primary site for sensing gravity. Amyloplasts within columella cells sediment in response to gravity and are thought to exert pressure on the endoplasmic

reticulum which then signals the roots growth direction (Boonsirichai et al., 2002). When the root tip hits an obstacle, (under experimental conditions, this is usually the agar surface), the pattern of cell expansion in the elongation zone alters, causing the root tip to change direction and grow either to the right or left of its original trajectory (Okada and Shimura, 1990; Simmons et al., 1995A; Rutherford and Masson, 1996; Sedbrook et al., 2002, 2004; Massa and Gilroy, 2003). This process sometimes is accompanied by axial rotation of the epidermal cell files or cell file rotation (CFR) in the elongation zone (Okada and Shimura, 1990). As mentioned before, different *Arabidopsis* ecotypes have different skewing angles. Roots of Col seedlings grow almost straight down (~6°) while roots of WS and Ler roots always skew to the left by ~20° (Rutherford and Masson, 1996). Observation of the CFR within the elongation zone is consistent with the root skewing angle; CFR is weaker in Col roots and is enhanced in WS and Ler (Rutherford and Masson, 1996; Simmons et al., 1995b). Many questions remain about the mechanism of root skewing. First, what is the relationship between root skewing and cell file rotation (CFR)? Although root skewing is associated with CFR, there is some evidence that CFR is not necessary for skewing (Buer et al., 2003). For example, no CFR could be observed despite strong skewing when wild type *Arabidopsis* was grown on plates containing half-strength Okada and Shimura salts and 1.5% (w/v) Bacto-agar (Migliaccio and Piconese, 2001; Buer et al., 2003). Second, what is the relationship between root waving and root slanting? Root waving is another root behavior in which root growth on an agar surface resembles a series of undulations. Some researchers believe that root skewing and waving share common steps involving both touch stimuli and gravity (Migliaccio and Piconese, 2001). In contrast, other researchers think root skewing and waving are two separate

processes. For example, root waving was detected in wild type roots treated with exogenous ethylene but no skewing was observed (Buer et al., 2003).

Several factors affect the root skewing process. First, different *Arabidopsis* ecotypes have different but consistent skewing angles when grown on vertically-oriented plates. Wild type Columbia (Col) seedling roots grow almost straight, skewing only about 6° to the left when viewed from top of the plate (Rutherford and Masson, 1996). On the other hand, wild type Wassilewekija (WS) and Landsberg (Ler) roots always skew about 20° to the left (Rutherford and Masson, 1996). Second, the amplitude of skewing is related to the age of the seedlings. *Sku* mutants have exaggerated right-slanting root growth compared to wild type when grown on plates tilted at a 30° angle. This deviation from the vertical in root-growth direction appeared very early after germination and increased over time. By the end of the tenth day of growth, the roots of *sku* mutants grew horizontally and some even grew upward (Rutherford and Masson, 1996). Third, the agar concentration of the medium influences the amplitude of skewing. On medium made with 0.8% or 1.5% agar, roots of *sku* mutants on tilted plates skew severely while on 0.4% agar medium the skewing is not significant suggesting that skewing is involved the interaction between the root and the agar surface (Rutherford and Masson, 1996). Fourth, application of cytoskeleton-altering drugs can regulate root skewing. Propyzamide and oryzalin are commonly used drugs that depolymerize microtubules (MTs). Since the orientation of the MTs determines the direction of epidermal cell elongation and since unequal epidermal cell elongation causes root skewing, propyzamide and oryzalin usually change the angle of root skewing, sometimes even to the opposite direction (Furutani et al., 2000; Sedbrook et al., 2004). Fifth, plant hormones are implicated in the root skewing

process. In the Ler ecotype, ethylene reduces the normal leftward skewing of roots. This suppression of root skewing is related to the inhibition of root elongation by ethylene (Buer et al., 2003). Sixth, root skewing is affected by certain nutrients in the medium such as sucrose. Adding sucrose to the medium does not alter root skewing with ethylene present. However, in the absence of exogenous ethylene, sucrose enhances root skewing (Buer et al., 2003). Seventh, salt affects microtubule organization, thus changing root growth patterns (Blancaflor and Hasenstein, 1995; Dhonukshe et al., 2003; Shoji et al., 2006). Evidence indicates that an exogenous high concentration of NaCl or KCl induces MT re-organization. For example, the MT array of cortical cells in maize roots reorients from transverse to parallel to the longitudinal axis in the presence of 350 mM KCl (Blancaflor and Hasenstein, 1995) and cortical MT arrays in tobacco By-2 cells rearrange from transverse orientation to a more random arrangement after treatment with 150 mM NaCl (Dhonukshe et al., 2003). A recent paper showed that a mutation in SOS1, a plasma membrane Na^+/H^+ antiporter, suppresses the phenotype of the *Spiral1* mutant, making the strongly right-skewing mutant roots grow almost straight down towards the direction of gravity and changing the rightward CFR to be parallel to the root organ axis (Shoji et al., 2006). The relationship between MT functions and sensitivity to salt is still unclear. One possibility is that this is an indirect effect caused by salt stress activating ABA and ethylene signaling pathways. Enhanced ethylene or ABA levels may destabilize MTs (Shibaoka, 1994; Achard et al., 2006). Another possibility is that phospholipase D (PLD) may be involved. PLD can function as a linker protein that helps bind MTs to membranes. When PLD was activated by Na^+, MTs were released and became free to rearrange (Gardiner et al., 2001, 2003; Dhonukshe et al., 2003).

As described in the previous chapter, I have identified 13 homozygous T-DNA mutant lines in all five *Arabidopsis* PP2A C subunit genes. No full-length mRNA was produced in 5 of these 13 homozygous mutant lines (*c1-1, c3-1, c3-2, c4-1* and *c4-2*). Wild type and *c1-1, c3-1, c3-2 and c4-1* mutant plants were compared under different growth conditions and chemical treatments to characterize the effect of the mutations. There was no obvious difference between growth of mutant and wild type plants except in one case: *c4-1* differed from wild type in the presence of NaCl. Here I investigate and further characterize this phenotype.

Materials and Methods

Growth on Medium Containing Supplemental NaCl

The effect of sodium concentration was examined by plating mutant and wild type seeds on medium supplemented with different concentrations of NaCl. Basic Medium contained 0.22% (w/v) Murashige & Skoog basal salt mixture with vitamins (Caisson Laboratories Inc., North Logan, UT) and 1% (w/v) washed agar, pH 5.7. The amount of Na^+ in this medium is 0.1 mM and the concentration of Cl^- is 3 mM. Additional NaCl (25 mM, 50 mM or 75 mM) was added to the Basic Medium to make the three types of NaCl media. For all experiments, wild type seeds and PP2A mutant seeds were sterilized with ethanol (see Chapter II) and placed on plates containing Basic Medium. After stratification, plates were kept in standard growth conditions (see Chapter II) and oriented vertically for 3 days. After 3 days, healthy seedlings were transferred to plates with different concentrations of NaCl. Two to three plates of each concentration were included in each experiment. Each experiment was repeated two or three times. Seedlings were placed in a straight row with their root tips aligned to a horizontal line marked on the square plates (13 cm x 13 cm, Fisherbrand). Five to eight seedlings of each genotype were transferred, depending on the experiment. Plates were placed vertically under standard growth conditions for another 7 days. Digital images of the plates were taken and root curvature was measured as the angle of deviation from the initial location of the seedling root tip to the final location of the root tip after 7 days of growth. At least 25 seedlings of each genotype were used in each treatment. Statistical differences were determined using the SYSTAT program (Chicago, IL).

Growth on Medium Containing Other Supplements

For some experiments Basic Medium or Basic Medium was supplemented with NaCl or other chemicals. After autoclaving, one of the sterile supplement solutions listed in Table 13 was added into the medium before pouring plates. Methods of sterilization and growing seeds were described in the methods section of Chapter II. Phenotype screening and measurements were described in the previous section.

Plasmid Construct for Complementation

A 5765 bp genome DNA fragment from the *Arabidopsis* wild type PP2A *C4* gene was amplified by PCR. The PCR product spanned from 1.8 kb upstream from the translational start site to 0.57 kb downstream from the translational stop site (Figure 28). For PCR, primers were designed using the program Primer Design (Lasergene, Madison, WI). The upper primer (C4Complement-F) was 5′ CTGTTCCGTAGGTGTGG3′ and the lower primer (C4Complement-R) was 5′CTATGCGAGTTTTTGAGC3′. All primers were synthesized by Integrated DNA Technologies (IDT, Coralville, IA). PCR was performed with ExTaq DNA polymerase (Takara, Moutain View, CA) in a PTC-100 programmable thermal controller (MJResearch, Watertown, MA). Each reaction contained of 12.5 μl of water, 2 μl of 10x ExTaq buffer (Takara, Moutain View, CA), 4 μl of 2.5 mM dNTP mix (Roche, Indianapolis, IN), 0.25 μl of both the forward and reverse primers at 12 pmol/μl, and 1 μl of wild type *Arabidopsis* ecotype Columbia template DNA at 150 ng/μl. A hot start cocktail was added to each reaction after the temperature reached 95°C. Each aliquot of the hot start cocktail included 4.25 μl of water,

Table 13. Medium supplements used in phenotype screening of PP2A C subunit mutants.

Supplements	**Concentration Added to Basic Medium**
KCl	50 mM
$CaCl_2$	10 mM
LiCl	5 mM
$NaNO_3$	50 mM
2,3,5-triiodobenzoic acid (TIBA)	1 μM
Propyzamide	3 μM
Cantharidin	10 μM

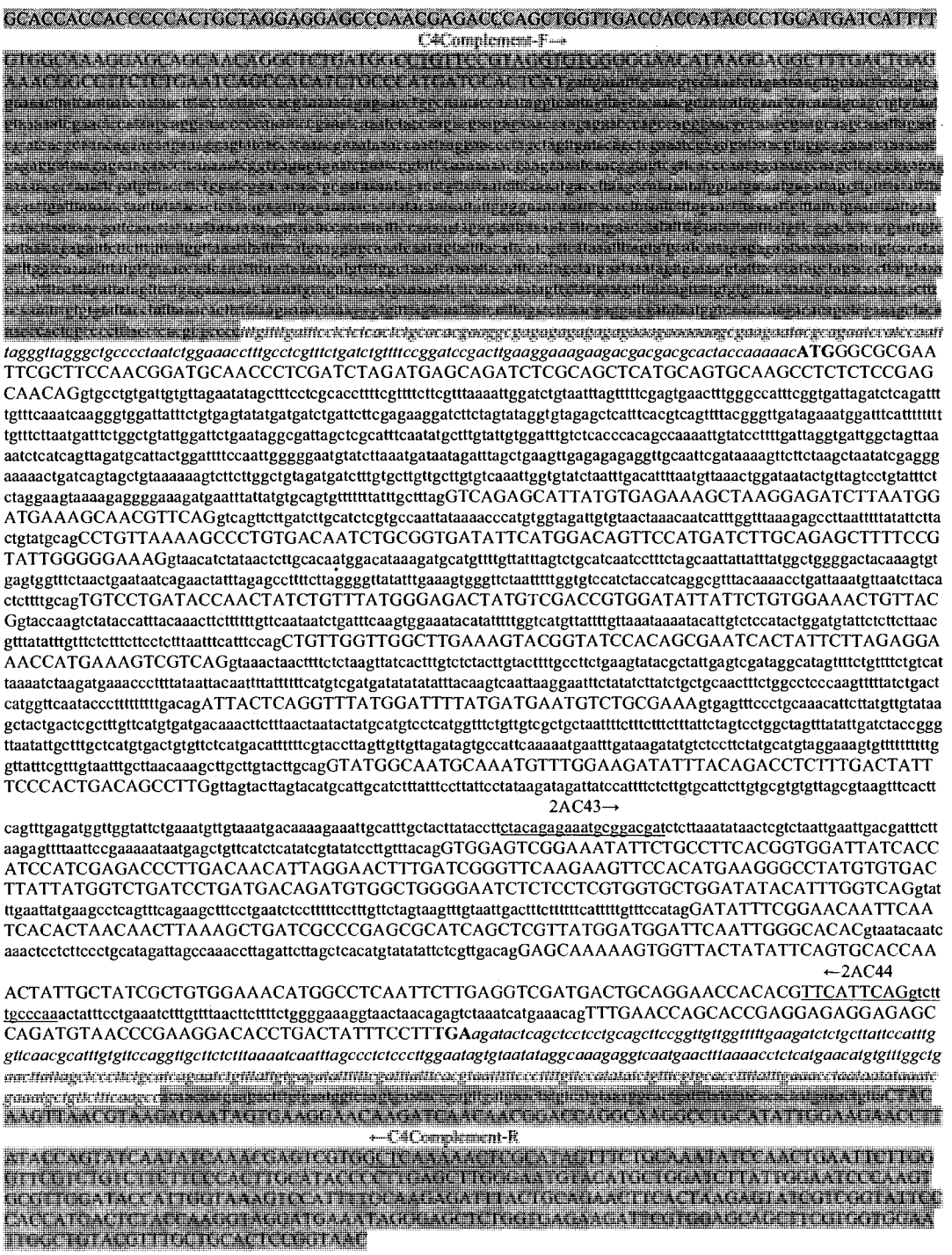


Figure 28. *Arabidopsis* PP2A *C4* genomic sequence (At3g58500). The start and stop codons are in bold. The coding sequence is in capitals on white background. The introns are in lower case letters on white background. The 5′ UTR and 3′ UTR sequences are in italics. The remaining genomic sequence is on a grey background in which coding sequence from the gene next to the PP2A C4 subunit gene is in capitals. The locations of primers used for PCR are underlined.

0.5 µl of 10x ExTaq buffer and 0.25 µl of ExTaq DNA polymerase. PCR was performed according to the following program: hot start at 94°C for 5 minutes, followed by 36 cycles at 94°C for 30 seconds, 50°C for 30 seconds and 72°C for 6 minutes. The program finished at 72°C for 5 minutes. PCR products were separated on 0.7% low-melt agarose, rapidly excised under UV light, and purified with the PCR Purification System (Promega, Cat. No. A7170, Madison, WI). Two separate PCR replicates were conducted to obtain independent clones; the PCR products were not sequenced to check for errors.

The two independent PCR products were cloned into pCR8/GW/TOPO which contains the spectinomycin resistance gene for selection in *E. coli* (Invitrogen, Cat. No. 45-0642). The cloning protocols followed the manufacturer's recommendations. Chemical transformation was performed with One Shot chemically competent *E. coli* TOP10 cells (Invitrogen, Cat. No. C404003, Carlsbad, CA). Chemical transformation was performed following the manufacturer's instructions. About 50 ng DNA was added to a vial of One Shot cells and mixed gently. The cells were incubated on ice for 30 minutes and then heat-shocked for 30 seconds at 42°C. The cells were put on ice for 2 minutes and 250 µl of pre-warmed LB medium [1% (w/v) tryptone, 0.5% (w/v) yeast extract and 1% (w/v) sodium chloride] was added. The tube was shaken horizontally at 37°C for 1 hour at 225 rpm and cells were spread on an LB plate containing 100 µg/ml spectinomycin. Plasmid was isolated and the correct clones were identified by digestion with *Eco*RI.

Once the entry vector was ready, an LR reaction was performed using Gateway LR Clonase II Enzyme Mix (Invitrogen, Cat. No. 11791-020) following manufacturer's

instructions. The destination vector pMDC123 (Curtis and Grossniklaus, 2003) contains two resistance genes: BASTA resistant gene (*BAR*) for plant selection and kanamycin resistant gene (*NPTII*) for bacterial selection. The LR reaction contained 103 ng of entry vector, 150 ng of pMDC123 Gateway destination vector, 8 µl of TE buffer (10 mM Tris-Cl and 1 mM EDTA, pH 8.0) and 2 µl of LR Clonase II enzyme. The reaction was incubated at 25°C for 1 hour. The reaction was stopped by adding 1 µl of Proteinase K (2 µg/µl) solution and incubating for 10 min. Chemical transformation was performed with One Shot chemically competent *E. coli* TOP10 cells (Invitrogen, Cat. No. C404003, Carlsbad, CA) as described. The clones were grown on LB plates containing 100 µg/ml kanamycin. Plasmid was isolated and the correct clones were identified by digestion with *Hin*dIII. The two constructs generated from the two replicate PCRs were named pC4-gen-1 and pC4-gen-2.

Agrobacterium Transformation

Agrobacterium tumefaciens strain GV3101 was used for plant transformation. Fifty ng of either pC4-gen-1 or pC4-gen-2 plasmid DNA was transformed into electrocompetent *Agrobacterium* cells. Cells were spread on plates containing 100 µg/ml of kanamycin and incubated at 28°C for 3 days. One colony was touched with a toothpick and transferred to 10 µl of water to use as template DNA for PCR to confirm that the colony contained the correct construct. For PCR, the upper primer 2AC43 was 5′CTACAGAGAAATGCGGACGAT3′ and the lower primer 2AC44 was 5′TTGGGCAAAGACCTGAATGAA3′ which were synthesized by Integrated DNA Technologies (IDT, Coralville, IA). The primers are shown in Figure 28. PCR was

performed in a PTC-100 programmable thermal controller (MJResearch, Watertown, MA). Each reaction contained of 6.2 μl of water, 1.5 μl of 10x ExTaq buffer (Takara, Moutain View, CA), 1.2 μl of 2.5 mM dNTP mix (2.5 mM each; Roche, Indianapolis, IN), 0.4 μl of both the upper and lower primers at 12 pmol/μl, 5 μl of *Agrobacterium* cell suspension, and 0.3 μl of home-made Taq DNA polymerase. PCR was performed according to the following program: hot start at 94°C for 5 minutes, followed by 36 cycles at 94°C for 30 seconds, 58°C for 30 seconds and 72°C for 1 minute. The program finished at 72°C for 5 minutes. PCR products were separated on a 1% agarose gel.

Plant Transformation and Selection

Transgenic plants were generated by a floral dip method (Clough and Bent, 1998) using *c4-1* mutant plants. About 10 wild type seeds per pot were grown in artificial growth medium [perlite (Whittemore Company, Inc, Lawrence, MA) and Metromix 360 (SUN GRO Horticulture, Bellevue, WA; 1:1] at 21°C, 16 hour photoperiod, and 100 μmol m^{-2} s^{-1} fluorescent light until bolts appeared (3-4 weeks). Primary bolts were clipped and plants were grown until secondary bolts appeared. A starter culture was prepared by inoculating *Agrobacterium tumefaciens* strain GV3101 carrying either of the PP2A C4 genomic constructs into 2 ml LB medium containing 100 μg/ml kanamycin. The culture was shaken at 28°C at 200-250 rpm for 24 hours or more. Two hundred ml of LB medium containing 100 μg/ml kanamycin was inoculated with the entire 2 ml starter culture and shaken overnight at 200-250 rpm at 28°C. Cells were harvested by centrifugation at 4,000 x g for 20 minutes at room temperature and resuspended in 5% (w/v) sucrose to an O.D. to 0.800±0.200 at 600 nm. Before dipping, Silwet L-77 (Lehle

Seeds, Cat. No. VIS-02, Round Rock, TX) was added to a final concentration of 0.05%. Flower stalks were dipped in the *Agrobacterium* suspension with gentle agitation for 10-20 seconds. The pots were laid on their sides overnight in a flat covered with a plastic dome to maintain humidity. The next day, plants were rinsed with water and grown at 21°C, 16 hour photoperiod, and 100±10 μmol m^{-2} s^{-1} of fluorescent light. The transformation procedure was repeated 5-7 days later to transform more young flower buds. After approximately 3-4 weeks, all T_1 seeds from one pot were harvested together.

T_1 seeds were planted in flats. The flats were kept in the dark at 4°C for 3-4 days and then moved to standard growth conditions of 21°C, 16 hour photoperiod, and 100 μmol m^{-2} s^{-1} fluorescent light. After 7-10 days, the seedlings were sprayed with Basta (240 μg/L) and grown for another 7-10 days until dark green resistant seedlings could be distinguished from the yellow dying seedlings. The healthy seedlings were transplanted into individual pots. Basta-resistant plants were confirmed to contain the correct transgene using the rapid PCR method described in Chapter II. Transformed seedlings were grown in a standard growth condition until T_2 seeds were ready to harvest.

T_2 seeds were grown on Basic Medium containing supplemental NaCl for the NaCl assay described earlier in this chapter. DNA was isolated using the rapid DNA isolation method described in Chapter II. PCR was conducted to detect the wild type *C4* transgene in the T_2 seedlings. For PCR, the upper primer was 2AC43 and the lower primer was 2AC44. PCR was performed in a PTC-100 programmable thermal controller (MJResearch). Each reaction contained 6.3 μl of water, 1 μl of 10x home-made buffer, 1.2 μl of 2.5 mM dNTP mix (Roche, Indianapolis, IN), 0.25 μl of both the forward and

reverse primers (IDT) at 12 pmol/μl, and 1 μl of template DNA from rapid DNA isolation for a total of 10 μl. A hot start cocktail was added to each reaction after the temperature reached 95°C. Each aliquot of the hot start cocktail included 4 μl of water, 0.5 μl of 10x ExTaq buffer and 0.5 μl of home-made Taq DNA polymerase. PCR was performed according to the following program: hot start at 94°C for 5 minutes, followed by 36 cycles at 94°C for 30 seconds, 58°C for 30 seconds and 72°C for 1 minute. The program finished at 72°C for 5 minutes. PCR products were separated on a 1% agarose gel.

Results

PP2A *c4* Mutants Exhibit Root Skewing in the Presence of NaCl

As described in Chapter III, phenotype screens showed no obvious differences between wild type and the *c1-1, c3-1, c3-2* and *c4-1* mutant plants with most of the growth conditions and chemical treatments tested. However, as described below, the *c4-1* mutant plants exhibited a root phenotype in the presence of NaCl when compared with wild type.

Seeds of wild type and *c4-1* mutant plants were plated on Basic Medium containing one-half strength MS, pH 5.7, and 1% agar. The amount of Na^+ in this medium is 0.1 mM and the concentration of Cl^- is 3 mM. After stratification, the plates were placed at 25°C with a 16-hr photoperiod. After three days, healthy seedlings were transferred to the same medium with or without 75 mM supplemental NaCl and grown for another 7 days. When viewed from the top of the plate, roots of wild type plants grown on Basic Medium showed a slight left skewing as did the *c4-1* mutant plants. In this study, I define root skewing as a transient change in root growth direction, after which roots appear to resume equal elongation of cells on both sides of the root. The average root skewing angle of wild type root was -13.9° and for *c4-1*, the root skewing angle was -12.2° (Figures 29 and 30). Left skewing is indicated by negative values and right skewing by positive values when seedlings were viewed from the top of the plates. *c4-1* mutants were compared with wild type in root angle and statistical analysis

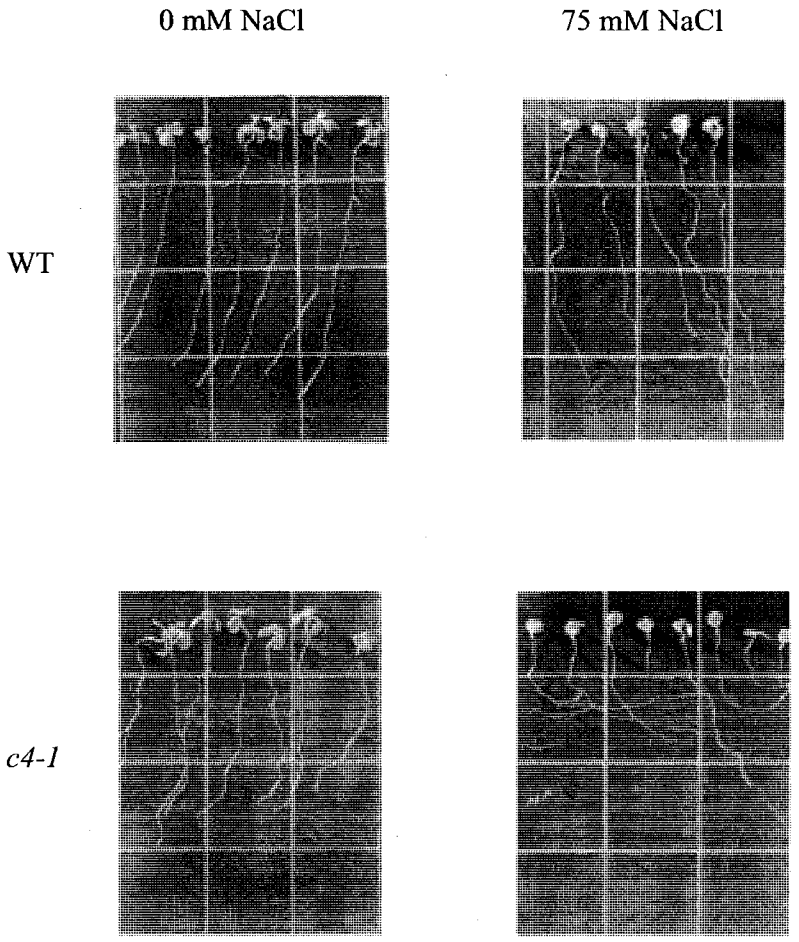


Figure 29. Root skewing of wild type (WT) and PP2A *c4-1* mutant seedlings. Seedlings were grown in medium with no supplemental NaCl for 3 days and then transferred to medium with or without supplemental 75 mM NaCl for 7 days.

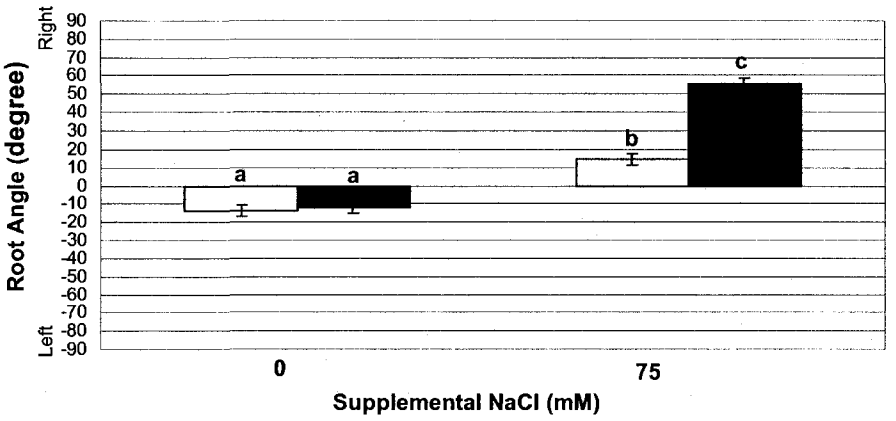


Figure 30. Root skewing on medium with supplemental NaCl. Wild type (□) and PP2A *c4-1* mutant (■) seedlings (n≥25) were grown on Basic Medium for 3 days, then seedlings were placed on medium containing 0 mM or 75 mM NaCl and grown for an additional 7 days. Root skewing angles were measured. Data was analyzed using ANOVA in all possible two way combinations. Standard error = 3.128.

indicated that there was no significant difference between wild type and the *c4-1* mutant seedlings (p=1.0). When 75 mM supplemental NaCl was added to the Basic Medium, the concentration of Na^+ in this medium is 75.1 mM and the concentration of Cl^- is 78 mM. On NaCl–supplemented medium, the roots of wild type skewed slightly to the right. The average root skewing angle was 14.1°. Statistical analysis was conducted to compare wild type roots grown with and without supplemental 75 mM NaCl. When compared with seedlings on non-NaCl supplemented plates, roots of wild type seedlings on supplemental 75 mM NaCl showed a significant difference in root angle from the non-supplemented wild type roots ($p \leq 0.01$). In the presence of 75 mM supplemental NaCl, the *c4-1* mutant plants also skewed to the right. However, the skewing angles of the *c4-1* mutants were much greater than the wild type. The average root skewing angle in wild type was 14.1° compared to 55.8° for the *c4-1* mutant (Figures 29 and 30). Statistical analysis indicated that there was a significant difference in root angle between wild type and PP2A *c4-1* mutant roots in the presence of 75 mM NaCl ($p \leq 0.01$). A NaCl concentration of 75 mM was chosen for this assay because, at NaCl concentrations less than 75 mM, skewing angles were not as great (details described later), while at NaCl concentrations greater than 75 mM, all seedlings looked unhealthy with yellow leaves and slow or no growth of roots (data not shown).

Other PP2A C Subunit Mutants Do Not Exhibit Strong Root Skewing in the Presence of NaCl

After identifying a root skewing phenotype for the PP2A *c4-1* mutant, the next question was whether other PP2A C subunit mutants exhibited a similar phenotype. I

have also identified plants with mutation in the *C1, C2, C3* and *C5* genes (see Chapter II). Alleles *c1-1, c3-1* and *c3-3* are knockouts; alleles *c2-1, c3-2, c4-3, c4-4, c4-5, c4-6, c5-1* and *c5-2* are not knockouts. Seedlings of all knockout mutant lines (alleles *c1-1, 3-1, c3-3, c4-1* and *c4-2*) as well as several non-knockout mutant lines (alleles *c2-1*, *c5-1* and *c5-2*) were tested for root growth in the presence of supplemental NaCl. I did not test root growth in the presence of supplemental NaCl on alleles *c3-2, c4-3, c4-4, c4-5* and *c4-6* since there are true knockout mutant plants (alleles *c3-1, c3-3, c4-1* and *c4-2*) available (Chapter II, Tables 8 and 9). I tested root growth in the presence of supplemental NaCl on the *c2-1*, *c5-1* and *c5-2* mutant plants because no true knockout mutant plants with mutations in either the *C2* or *C5* genes was available. These plants served as additional controls to test the possibility that a gene on the T-DNA might be responsible for the root skewing phenotype. When viewed from the top of the plates, roots of all of the tested C subunit mutants showed a slight left skewing similar to wild type on medium containing no supplemental NaCl (Figure 31). When grown on medium with 75 mM supplemental NaCl, the roots of both mutant and wild type plants skewed to the right. However, only two mutants, *c4-1* and *c4-2*, showed a strong right skewing phenotype (Figure 31 and Table 14). Statistical analysis was conducted to compare wild type and individual PP2A mutants either on medium with no supplemental NaCl or on medium with supplemental 75 mM NaCl. ANOVA indicated that there was no significant difference between root angles of wild type and any of the PP2A mutants on Basic Medium with no supplemental NaCl ($p>0.9$). However, in the presence of 75 mM supplemental NaCl, there were significant differences between the skewing angles of both *c4* mutants when compared to

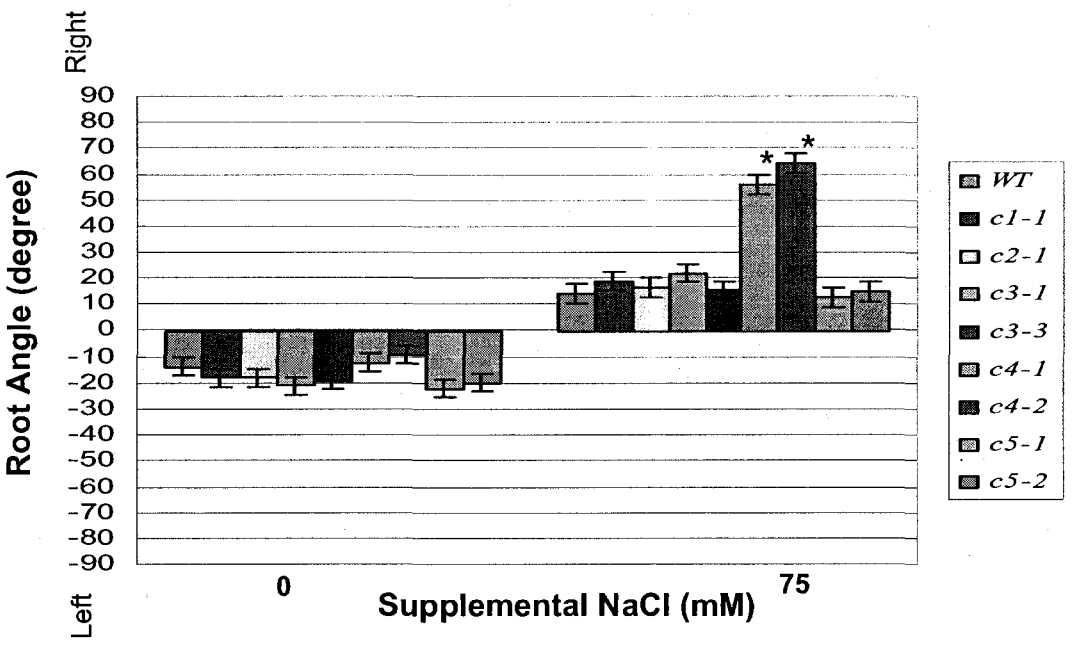


Figure 31. Root skewing of wild type (WT) and PP2A C subunit mutant seedlings on medium with supplemental 75 mM NaCl. After growth on Basic Medium for 3 days, seedlings (n>25) were placed on medium containing 0 mM or 75 mM NaCl and grown for an additional 7 days. Root skewing angles were measured. Data were analyzed using ANOVA. Root skewing angles are shown in Table 14. SE = 3.522. * indicates a significant difference when comparing a mutant line to wild type at a particular NaCl concentration ($p \leq 0.01$). Alleles *c1-1, c3-1, c3-3, c4-1* and *c4-2* are knockouts; alleles *c2-1, c5-1* and *c5-2* are not knockouts.

Table 14. Average root skewing angles of wild type (WT) and PP2A C subunit mutant seedlings. Negative values indicate left skewing; positive values indicate right skewing. * indicates values significantly different from wild type in a given treatment.

Allele	Root Angle	
	No Supplemental NaCl	**75 mM Supplemental NaCl**
WT	-13.9°	14.2°
c1-1	-18.1°	19.0°
c2-1	-18.2°	16.3°
c3-1	-21.1°	21.8°
c3-3	-19.3°	15.4°
c4-1	-12.2°	55.8° *
c4-2	-9.3°	64.1° *
c5-1	-22.1°	12.4°
c5-2	-19.8°	14.7°

wild type ($p \leq 0.01$) while there was no significant difference between any of the other PP2A mutants and wild type on this medium ($p > 0.9$).

The Root Skewing Phenotype of *c4* Mutants is Dependent on NaCl Concentration

To test the effect of the NaCl concentration on root skewing, seeds of wild type and *c4-1* and *c4-2* mutants were placed on Basic Medium, stratified at 4°C for 3-4 days, and transferred to a growth chamber at 25°C with a 16-hr photoperiod. After three days, healthy seedlings were transferred to Basic Medium containing either no supplemental NaCl or 25, 50, or 75 mM supplemental NaCl and grown for another 7 days in vertically-oriented plates. The experiments were repeated twice. When viewed from the top of the plates, mutants grown on Basic Medium with no supplemental NaCl showed a slight leftward root skewing similar to wild type (Figure 32). As the concentration of supplemental NaCl added to the medium increased, the rightward skewing of roots of both wild type and mutant plants increased and was greatest at 50 mM and 75 mM supplemental NaCl (Figures 32 and 33A). Although a right skewing phenotype at these higher NaCl concentrations was observed in wild type seedlings, the skewing angles in the mutants were much greater compared with wild type (Figures 32 and 33A). At NaCl concentrations greater than 75 mM, all seedlings looked unhealthy with yellow leaves and slow or no growth of roots (data not shown), so 75 mM was the highest NaCl concentration that was routinely tested. The angles of root skewing were measured and statistical analysis was conducted. At 25 mM supplemental NaCl, the skewing angles of roots of mutant plants were about 20° greater than wild type (Table 15). At 50 mM and 75 mM, the skewing angles of mutant plant roots were about 50° greater than wild type

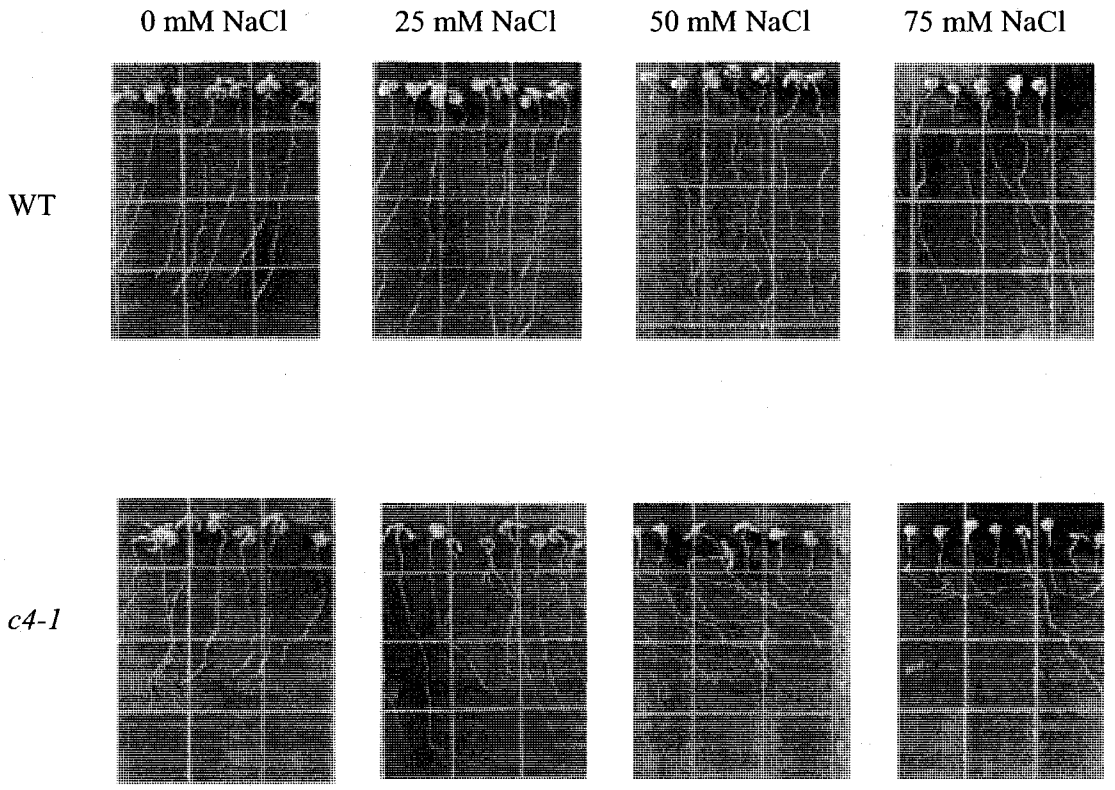


Figure 32. Root skewing of wild type (WT) and PP2A *c4-1* mutant seedlings at different NaCl concentrations. Seedlings were grown in medium with no supplemental NaCl for 3 days and then transferred to medium supplemented with different concentrations of NaCl for 7 days.

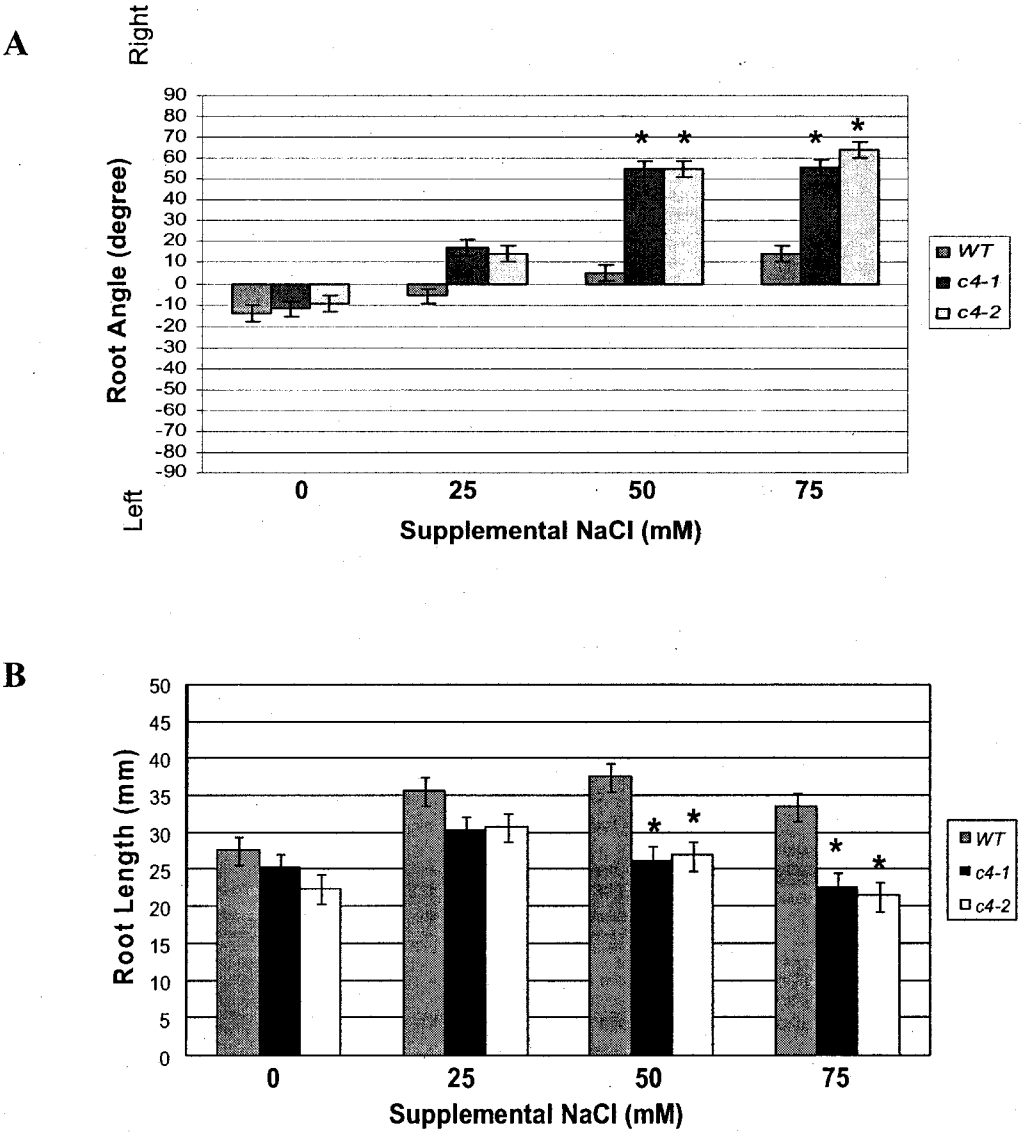


Figure 33. Root growth of wild type, *c4-1* and *c4-2* seedlings on different NaCl concentrations. After growth on Basic Medium for 3 days, seedlings (n>25) were placed on medium containing 0 mM, 25 mM, 50 mM, or 75 mM supplemental NaCl and grown for an additional 7 days. **A)** Root skewing angles and **B)** root length were measured. Data were analyzed using ANOVA. Average root skewing angles are shown in Table 15. Average root lengths are shown in Table 16. * indicates a significant difference when comparing a mutant line with wild type at a particular NaCl concentration ($p \leq 0.01$).

Table 15. Average root skewing angles of wild type (WT), *c4-1* and *c4-2* mutant seedlings. Negative values indicate left skewing; positive values indicate right skewing. Data were analyzed using ANOVA. * indicates values significantly different from wild type in a given treatment. Standard error = 3.8.

		Average Root Angle		
		WT	*c4-1*	*c4-2*
NaCl Concentration (mM)	**0**	-13.9°	-12.2°	-9.3°
	25	-6.1°	17.0°	14.3°
	50	4.7°	54.6° *	55.0° *
	75	14.1°	55.8° *	64.1° *

and were significantly different from wild type ($p \leq 0.01$; Figure 33A and Table 15). These experiments indicated that the degree of root skewing of both wild type and *c4* mutant seedlings was dependent on NaCl concentration and that the rightward skewing of *c4* mutant roots was significantly greater than wild type at 50 mM and 75 mM NaCl.

Root length was also measured in this experiment (Figure 33B and Table 16). There was a general trend that the length of mutant plant roots appeared shorter than wild type but root length of *c4-1* and *c4-2* mutants were significantly different from wild type only at 50 mM and 75 mM ($p \leq 0.05$; Figure 33B and Table 16). Reduced root growth under NaCl treatment was reported for the PP2A *a1* subunit mutant (*rcn1*; Blakeslee et al., 2008). Root lengths of *a1* mutant seedlings were significantly shorter than wild type at 50 mM, 75 mM and 100 mM NaCl. Since PP2A *c4* and *a1* mutants exhibited a similar decrease in root length in the presence of NaCl, these results indicate that a PP2A heterodimer or heterotrimer containing the A1 and C4 subunits may play an important role in responding to NaCl stress in seedling roots.

The *c4* Mutant Phenotype is Due To Loss of PP2A C4 Function

To confirm that the disruption of the PP2A *C4* gene caused the *c4* mutant phenotype, a complementation test was conducted. A 5765 bp fragment of genomic DNA was amplified by PCR and cloned into the transformation vector pMDC123 which contains a Basta-resistance gene. This DNA fragment contained the complete *C4* genomic coding region as well as ~ 1.8 kb of *C4* upstream sequences and ~ 0.86 kb of downstream sequences. Since the exact location of the promoter and terminator of the *C4*

Table 16. Average root length of wild type, *c4-1* and *c4-2* mutant seedlings. Data were analyzed using ANOVA. * indicates values significantly different from wild type in a given treatment. Standard error = 2.3.

		Average Root Length (mm)		
		WT	*c4-1*	*c4-2*
NaCl Concentration (mM)	**0**	27.7	25.3	22.5
	25	35.7	30.4	30.7
	50	37.6	26.3 *	26.9 *
	75	33.6	22.7 *	21.4 *

gene is unknown, the cloned fragment contained all the genomic sequence between *C4* and the adjacent genes. Two independent PCR replicates were cloned and these two constructs are named pC4-gen-1 and pC4-gen-2. T_2 seeds were generated following transformation of *Arabidopsis* T-DNA *c4-1* mutants. Each set of T_2 transformants was analyzed for root skewing on medium with and without supplemental NaCl. Plants were also transformed with empty pMDC123 vector as a control. If complementation occurs, due to the segregation of the transgene in the T_2 generation, about 75% of the T_2 seedlings were expected to show the complemented (wild type) phenotype of slight rightward skewing on NaCl medium, while about 25% of the T_2 seedlings should show the strong root skewing phenotype of the *c4-1* mutants because the *C4* transgene is no longer in their genome. On medium without supplemental NaCl, all the T_2 seedlings were expected to show a slight left skewing phenotype. The root skewing phenotypes of T_2 seedlings were scored and the percentage of seedlings with the wild type phenotype is shown in Table 17. About 70% of seedlings from two independent T_2 families showed complementation of the *c4* phenotype in the root skewing assay in the presence of supplemental NaCl as demonstrated by only a slight rightward root growth (Figure 34A). There was no significant difference between expected results and observed results ($p>0.5$). PCR was used to check the transgenic plants for the presence of the wild type *C4* transgene. Seedlings with both skewing and non-skewing phenotypes were selected and used for rapid DNA isolation. PCRs were performed with two C4 primers which span the T-DNA insertion site. As expected, no PCR product was obtained from seedlings with the strong root skewing phenotype indicating that there is no *C4* transgene present in their

Table 17. Phenotypes of T_2 seedlings following transformation of *c4-1* mutant plants with the *C4* wild-type gene. Two independent T_2 lines containing the T-DNA from pC4-gen-1 and pC4-gen-2 were scored, as well as plants with the T-DNA containing no *C4* gene (empty vector). Data was analyzed by the Chi Square test.

Construct	NaCl Concentration (mM)	Expected % wild type root phenotype in T_2 generation	Observed % wild type root phenotype in T_2 generation
pC4-gen-1	0	100	100 (n=74)
	75	75	70 (n=80)
pC4-gen-2	0	100	100 (n=79)
	75	75	71(n=72)
Empty Vector	0	0	0 (n=40)
	75	0	3 (n=40)

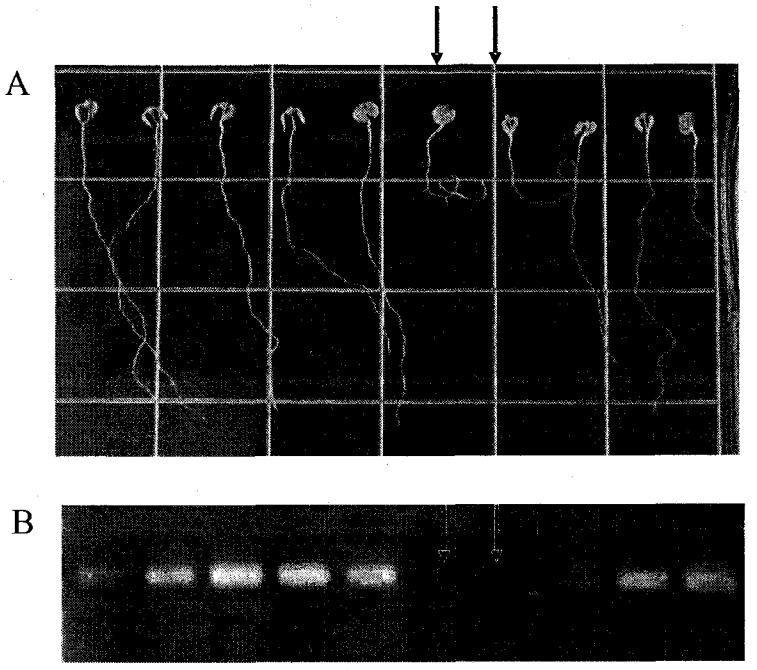


Figure 34. Characterization of T_2 *c4-1* seedlings segregating for the wild type *C4* transgene. **A.** Following transformation with a wild type *C4* transgene from pC4-gen-1, T_2 seedlings were grown on Basic Medium for 3 days, transferred to medium containing 75 mM supplemental NaCl and grown for an additional 7 days. Arrows indicate seedlings which did not show the wild type root phenotype. **B.** PCR to identify seedlings carrying a wild type *C4* transgene. Arrows indicate the reactions that did not amplify the wild type *C4* gene.

genome (Figure 34B). A *C4* gene product was observed with the DNA template from seedlings with a non-skewing (wild type) phenotype indicating that their genome contains the *C4* transgene (Figure 34B). These results show that the wild type *C4* gene complements the *c4* root skewing phenotype.

A PP2A Inhibitor Phenocopies the C4 Mutant Phenotype

To test whether a PP2A inhibitor can mimic the root skewing phenotype of *c4* mutant plants, wild type seedlings were grown on medium containing both cantharidin and NaCl. Cantharidin is a PP2A inhibitor that has previously been used to mimic the effect of a PP2A mutation in *Arabidopsis* (Deruere et al., 1999; Rashotte et al., 2001). Two replicates of the experiment were done. When no cantharidin or NaCl was added, roots of wild type seedlings showed a slight left skewing as observed before (Figures 31 and 35). When only 75 mM supplemental NaCl was added, roots of wild type seedlings exhibited a slight right skewing as expected (Figure 35). When only 10 μM cantharidin was added, roots of wild type seedlings showed a slight left skewing as well as shorter root length. Inhibition of root growth by cantharidin has been observed previously (Figure 35; Deruere et al., 1999; Zhou et al., 2004). Roots of wild type seedlings on medium containing both 10 μM cantharidin and 75 mM supplemental NaCl consistently produced a phenocopy of the strong right skewing phenotype observed for the *c4* mutants on NaCl (Figure 35). In fact, the root skewing phenotype of wild type grown on both 10 μM cantharidin and 75 mM supplemental NaCl (Figure 35) may even be stronger than the *c4* mutant in the presence of 75 mM supplemental NaCl (Figures 29 and 32). One possible explanation is that cantharidin does not inhibit the C4 subunit specifically; so

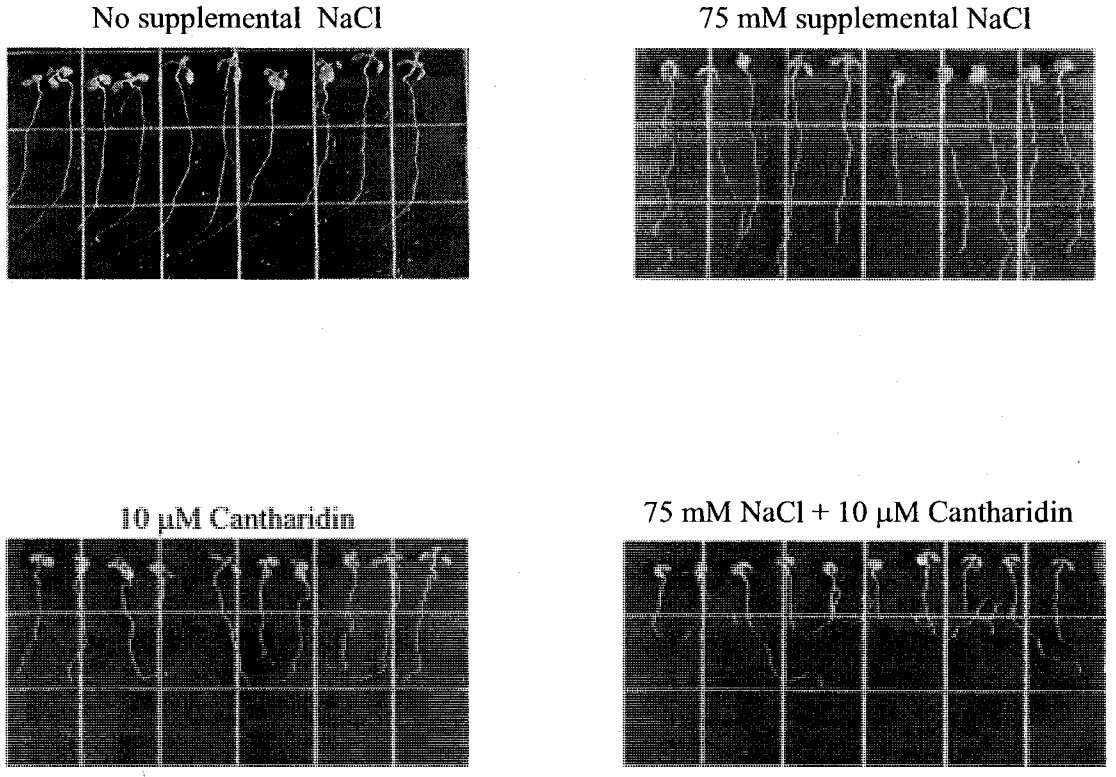


Figure 35. Effect of cantharidin and NaCl on root growth of wild type seedlings. After growth on Basic Medium for 3 days, seedlings (n=40) were placed on medium containing 0 mM or 75 mM supplemental NaCl with or without 10 μM cantharidin and grown for an additional 7 days.

there may be additional PP2A subunits that contribute to a lesser extent to root skewing that are also being inhibited by cantharidin. Since cantharidin is unstable in light, these experiments should be repeated using a yellow acrylic filter to block degradation of cantharidin (Baskin and Wilson, 1997); however, since the roots did exhibit skewing, it is likely that significant levels of cantharidin were still present. These data support the hypothesis that PP2A regulates the direction of root growth in the presence of NaCl.

Chloride is Not Important for Root Skewing

To examine whether the root skewing phenotype was caused by general osmotic stress or by the presence of specific ions, the effects of LiCl, KCl, $CaCl_2$ and $NaNO_3$ on the growth of the PP2A *c4* seedlings roots were analyzed. LiCl, KCl, $CaCl_2$ were used to compare with NaCl to test whether chloride is important for root skewing and $NaNO_3$ was chosen to test whether sodium is critical for root skewing. I chose 5 mM LiCl because this LiCl concetration was commonly used in published work (Mitsuya et al., 2005). I chose 50 mM supplemental KCl and 50 mM supplemental $NaNO_3$ because root skewing of the *c4* mutant was significantly different from wild type at 50 mM supplemental NaCl (Figure 33). I chose 10 mM $CaCl_2$ based on previously published work and because this experiment was also designed to test effect of Ca^{2+} on root skewing (details described later). Note that the final concentration of each ion is much higher than that present in Basic Medium (Table 18).

When *c4-1* and *c4-2* mutant seedlings were grown in the presence or absence of 5 mM LiCl, 50 mM KCl or 10 mM $CaCl_2$, a slight left skewing was observed in both *c4* mutant and wild type seedlings and there was no significant difference when comparing

Table 18. Comparison of concentration of selected ions in Basic Medium and supplemented medium.

Ion	Concentration in Basic Medium (mM)	Final Concentration in Supplemented Medium (mM)
K^+	10	60
Ca^{2+}	1.5	11.5
Li^+	0	5
Cl^-	3	5 - 50 (depending on counter ion)
NO_3^-	20	70
Na^+	0.1	50.1

the PP2A mutants and wild type within each treatment ($p>0.1$; Figure 36 and Table 19). Statistical analysis was also conducted to compare the effects of LiCl, KCl, and $CaCl_2$ on each genotype. There was no significant difference in root angle for seedlings grown on LiCl, KCl, or $CaCl_2$ for either wild type ($p>0.1$) or PP2A *c4* mutants ($p>0.07$). These results indicate that the root skewing phenotype was not caused by general osmotic stress and that chloride is not important for the root skewing response.

When no supplemental $NaNO_3$ was added to the medium, roots of both wild type and *c4* mutants showed a slight left skewing. There was no significant difference in root angle between PP2A mutant and wild type roots on Basic Medium in the absence of $NaNO_3$ ($p>0.1$), as observed previously. When 50 mM $NaNO_3$ was added to the medium, a slight rightward skewing phenotype was observed in wild type seedlings. There was a significant difference in the root angle of wild type seedlings with and without supplemental $NaNO_3$ ($p<0.01$). This result is similar to the effect of 75 mM supplemental NaCl on wild type. When the *c4* mutant seedlings were grown in the presence of 50 mM $NaNO_3$, roots showed a rightward skewing phenotype as observed in wild type seedlings. There was a significant difference in root angle of *c4* mutants with and without supplemental $NaNO_3$ ($p<0.01$). Statistical analysis was conducted to compare root angles of wild type and individual PP2A mutants in the presence of 50 mM $NaNO_3$. There was no significant difference in root angle between PP2A mutant and wild type roots ($p>0.1$). These results indicate that both wild type and *c4* mutant roots responded similarly to $NaNO_3$. Further data need to be collected to determine whether the Na^+ ion is the important factor for root skewing.

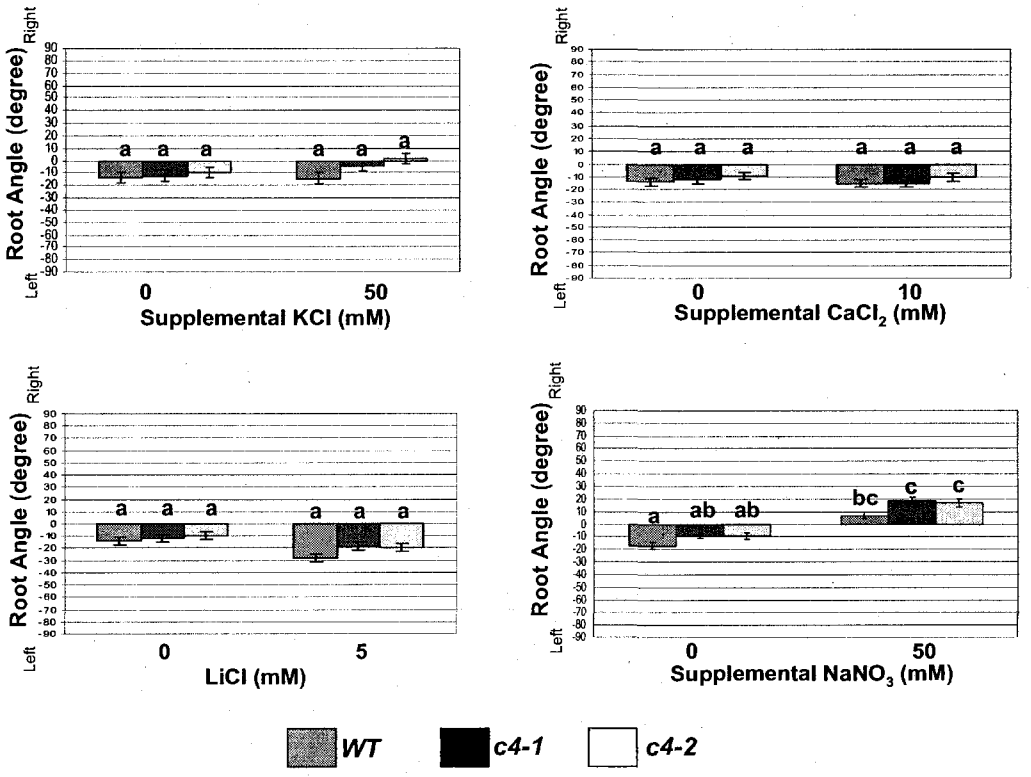


Figure 36. Root skewing in the presence of supplemental LiCl, KCl, $CaCl_2$ or $NaNO_3$. After growth on Basic Medium for 3 days, seedlings ($n \geq 25$) were transferred to medium containing no chemical supplement or supplemented with 50 mM KCl, 5 mM LiCl, 10 mM $CaCl_2$, or 50 mM $NaNO_3$ and grown for an additional 7 days. Root skewing angles were measured. Data were analyzed using ANOVA. Root skewing angles and standard error (SE) are shown in Table 19. Same letter denotes no significant difference ($p>0.07$). Different letter denotes a significant difference ($p<0.01$).

Table 19. Average root skewing angle in medium containing added of LiCl, KCl, $CaCl_2$ or $NaNO_3$. Negative values indicate left skewing; positive values indicate right skewing.

Supplement	Concentration (mM)	Average Root Skewing Angle			SE
		WT	*c4-1*	*c4-2*	
KCl	0	-13.9°	-12.2°	-9.3°	4.2°
	50	-14.2°	-4.8°	1.9°	
$CaCl_2$	0	-13.9°	-12.2°	-9.3°	3.1°
	10	-15.1°	-15.5°	-10.4°	
LiCl	0	-13.9°	-12.2°	-9.3°	3.3°
	5	-28.3°	-18.4°	-19.7°	
$NaNO_3$	0	-17.2°	-8.6°	-9.3°	2.9°
	50	7.0°	18.6°	16.6°	

$CaCl_2$ Decreases the Root Skewing Phenotype of PP2A *c4* Mutants

When plants are under NaCl stress, Na^+ displaces Ca^{2+} from the plasma membrane leading to a disruption of membrane integrity and increased membrane permeability (Cramer et al., 1985; Essah et al., 2003). External Ca^{2+} inhibits the initial Na^+ influx into cells and blocks Na^+ accumulation in cells, showing that Ca^{2+} plays a vital role in maintenance of membrane integrity (Cramer et al., 1985; Essah et al., 2003). To test the effect of Ca^{2+} on root skewing in the presence of NaCl, seeds of the PP2A *c4* mutants were sown on plates containing different concentrations of supplemental NaCl plus 10 mM supplemental $CaCl_2$. I chose 10 mM supplemental $CaCl_2$ following the procedure on *Arabidopsis* Gantlet Project website (http://thale.biol.wwu.edu/) and other published data. Ten mM supplemental $CaCl_2$ was in a concentration range commonly used (Mitsuya et al., 2005; Wang et al., 2007). Note that the final concentration of Ca^{2+} and Cl^- are much higher than that present in Basic Medium (Table 18). Both wild type and mutant seedlings exhibited similar trends in root growth in the presence of NaCl, whether or not $CaCl_2$ was added. As previously observed, wild type roots changed from slightly left skewing to slightly right skewing as the concentration of NaCl increased while mutants showed significant strong right skewing at 50 mM and 75 mM NaCl ($p<0.01$; Figure 37 and Table 20). Interestingly, a trend was observed in which supplemental $CaCl_2$ decreased the magnitude of rightward root skewing caused by NaCl in both wild type and PP2A mutants. To compare the effect of $CaCl_2$ at each NaCl concentration, statistical analyses were conducted. In both the *c4-1* and *c4-2* mutant lines, the decrease in root skewing on medium containing both supplemental 75 mM NaCl and

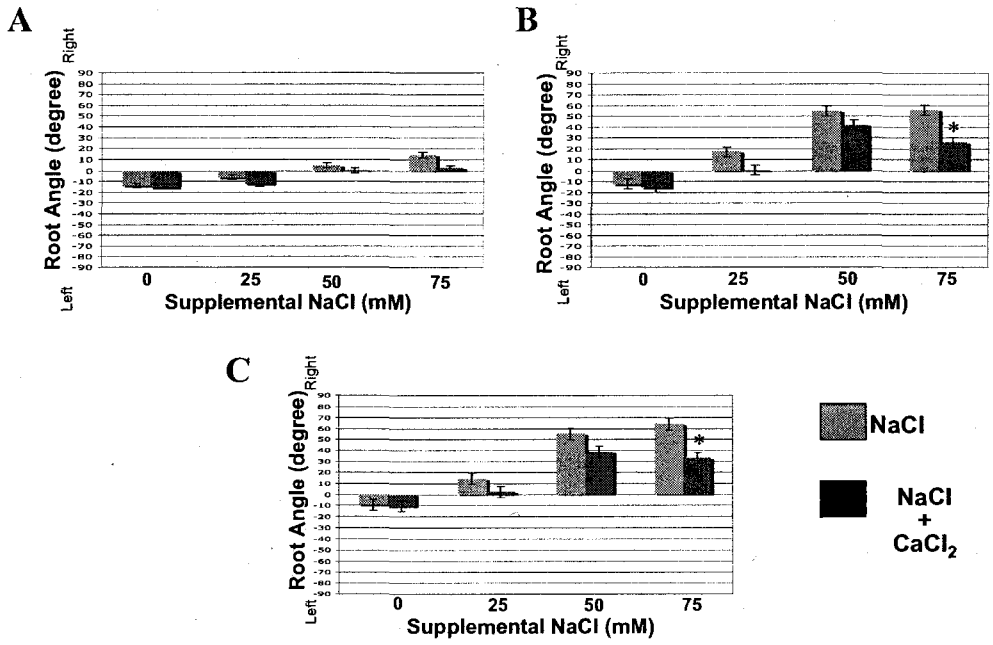


Figure 37. Root skewing in the presence of supplemental NaCl and $CaCl_2$. After growth on Basic Medium for 3 days, seedlings ($n \geq 25$) were transferred to medium containing no chemical supplement or supplemental 10 mM $CaCl_2$ plus 0 mM, 25 mM, 50 mM or 75 mM supplemental NaCl and grown for an additional 7 days. A) wild type; B) *c4-1*; and C) *c4-2*. Root skewing angles were measured. Data was analyzed using ANOVA. Root skewing angles and Standard Error are shown in Table 20. * denotes a significant difference when comparing the plus or minus supplemental $CaCl_2$ at a particular NaCl concentration in each genotype ($p \leq 0.01$).

Table 20. Average root skewing angle in the presence of NaCl and 10 mM $CaCl_2$. Negative values indicate left skewing; positive values indicate right skewing. * indicates values are significantly different when comparing the plus or minus supplemental $CaCl_2$ at a particular NaCl concentration in each genotype. SE = standard error.

NaCl (mM)	**10 mM $CaCl_2$**	**Average Root Angle**		
		WT	*c4-1*	*c4-2*
0	No	-13.9°	-12.2°	-9.3°
	Yes	-15.1°	-15.5°	-10.4°
25	No	-6.1°	17.0°	14.3°
	Yes	-12.4°	0.4°	2.2°
50	No	4.7°	54.6°	55.0°
	Yes	0.3°	41.7°	38.7°
75	No	14.1°	55.8°	64.1°
	Yes	2.6°	25.5° *	33.1° *
SE		2.2°	4.5°	5.2°

10 mM $CaCl_2$ was significant ($p \leq 0.01$; Figure 37 and Table 20). These results indicate that Ca^{2+} decreases root skewing in the presence of NaCl.

An Auxin Transport Inhibitor Causes a Root Curling Phenotype in the PP2A *c4* Mutant

The PP2A A subunit mutant *rcn1* was identified based on an altered root growth pattern in the presence of the non-competitive auxin transport inhibitor NPA which normally causes wild type roots to fail to curl when they contact the bottom of a plate (Garbers et al., 1996). To examine the effect of auxin transport on PP2A *c4* mutants, seeds of the PP2A *c4* mutants were sown on horizontally oriented plates containing 0 or 50 mM NaCl plus 5 μM NPA following the procedure described by Garbers et al. (1996). In contrast to the *rcn1* mutant, there was no visual difference between wild type and PP2A *c4* mutant plants in root growth (data not shown).

A second auxin transport inhibitor TIBA was tested using vertically-oriented plates. Seeds of the PP2A *c4* mutants were sown on plates containing 0 or 50 mM NaCl plus 1 μM TIBA. TIBA is a competitive auxin transport inhibitor. Fifty mM NaCl was chosen because roots of both wild type and mutant plants did not grow on medium containing 75 mM NaCl and 1 μM TIBA. As observed before, when viewed from the top of the plate, roots of both wild type and *c4* mutant seedlings grown on Basic Medium showed a slight left skewing (Figure 38). When 50 mM supplemental NaCl was added to the Basic Medium, the roots of wild type showed a slight right skewing. The *c4* mutant plants showed a stronger right skewing on 50 mM NaCl when compared with wild type

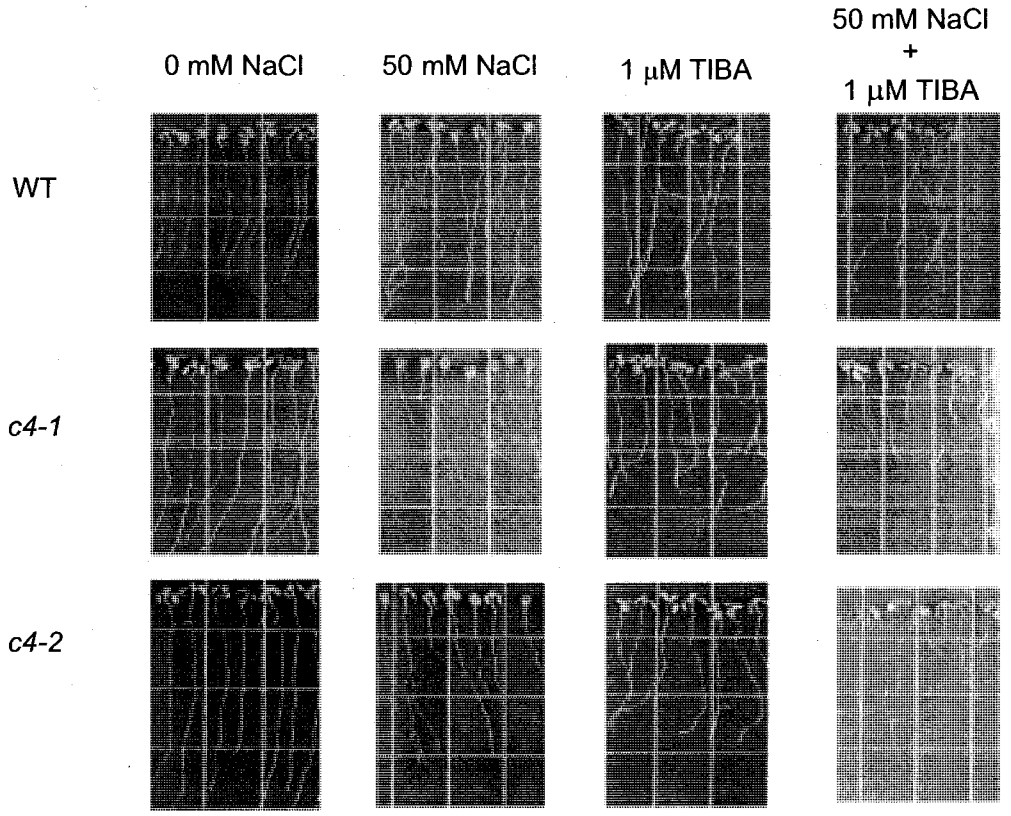


Figure 38. Root phenotype of wild type (WT) and PP2A *c4* mutants in the presence of 50 mM NaCl, 1 μM TIBA or both. After growth on Basic Medium for 3 days, seedlings were placed on medium containing combinations of 0 or 1 μM TIBA plus 0 mM or 50 mM NaCl, and grown for an additional 7 days.

plants as observed previously. In the presence of 1 μM TIBA alone, roots of both PP2A *c4* mutants and of wild type seedlings grew along the gravity vector and did not show any root skewing phenotype (Figure 38). As expected, by visual inspection, in the presence of 1 μM TIBA, roots of both wild type seedlings and *c4* mutant seedlings appeared shorter compared to seedlings grown on Basic Medium without TIBA as noted previously (Rahman et al., 2007). Surprisingly, many of the PP2A *c4* mutants exhibited a root curling phenotype on medium with 50 mM NaCl plus 1 μM TIBA, while no root curling occurred in wild type roots (Figure 38). In this study, I define root curling as a continuous change in the direction of root growth due to unequal elongation of cells on one side of the roots. These results suggest that the change of the direction of root growth observed in PP2A *c4* mutants in the presence of NaCl may involve changes in auxin transport. Furthermore, root curling phenotype in the presence of NaCl and TIBA in *c4* mutant was not observed in the presence of NaCl and NPA even though these two experiments were set up in different oriented plates, indicating that TIBA and NPA may act through different pathways. Similarly, Garbers also showed that root curling phenotype in *rcn1* mutant was not observed in the presence of TIBA (Garbers, et al., 1996). This result supports my hypothesis that TIBA and NPA may function in different pathway.

Wild Type and PP2A *c4* Mutants Respond Similarly to an Anti-microtubule Drug

The drug propyzamide can depolymerize microtubules leading to enhanced root skewing in wild type seedlings (Furutani et al., 2000; Yuen et al., 2003). To examine the effect of propyzamide on PP2A *c4* mutants, seeds were sown on plates containing 0 or 50

mM NaCl plus 3 μM propyzamide. I followed other published procedure choosing 3 μM propyzamide because propyzamide concentrations higher than 3 μM will inhibit root elongation and abolish skewing (Yuen et al., 2005). When grown in Basic Medium only, roots of both wild type and *c4* mutants displayed slight left skewing and when supplemental NaCl was added to the medium, roots of both wild type and PP2A *c4* mutants displayed right skewing as observed before (Figures 30 and 39). On medium with propyzamide alone, roots of both wild type and PP2A *c4* mutant seedlings showed a significant strong left skewing when viewed from the top of the plates ($p<0.01$; Figure 39 and Table 21). When both supplemental NaCl and propyzamide were added to the medium, the roots in both wild type and *c4* mutant seedlings displayed rightward skewing. However, roots of wild type plants displayed only a slight right skewing while *c4* mutants showed significant strong right skewing ($p<0.01$). Interestingly, the right skewing in the presence of NaCl and propyzamide was less strong in *c4* mutants than the skewing observed with NaCl alone but this effect was not significant ($p>0.1$). These results indicate that propyzamide and NaCl have antagonistic effects on root skewing.

PP2A *a1* Mutant Exhibits Root Curling in the Presence of NaCl

A PP2A A subunit mutant with a T-DNA insertion has been reported to have an altered root growth phenotype (Garbers et al., 1996). This *A1* subunit mutant was identified due to its root curling ability in the presence of naphthylphthalamic acid (NPA) which is an auxin transport inhibitor. In wild type plants, roots do not curl in the presence of NPA (Garbers et al., 1996). To test whether the *a1-1* mutant has a similar root growth phenotype in the presence of 75 mM supplemental NaCl to the *c4* mutants, seeds of wild

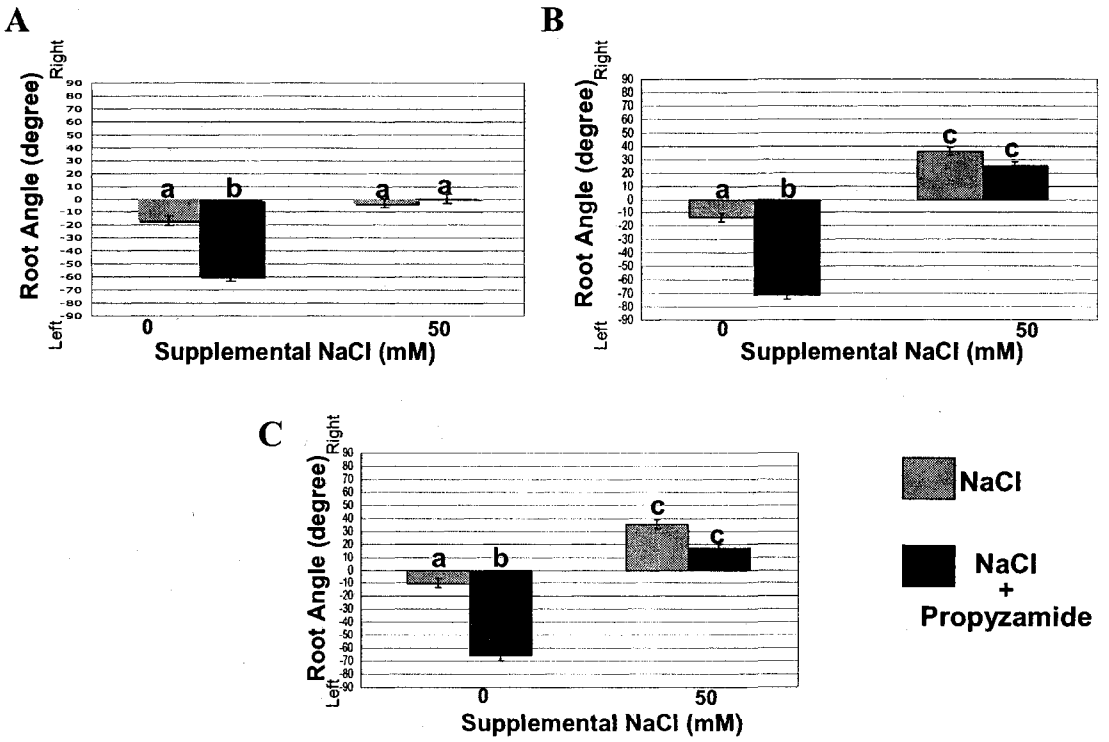


Figure 39. Root skewing phenotype in the presence of NaCl and propyzamide. After growth on Basic Medium for 3 days, seedlings (n≥25) were transferred to medium containing no chemical supplement or 3 μM propyzamide plus 0 mM or 50 mM supplemental NaCl and grown for an additional 7 days. **A)** wild type; **B)** *c4-1*; and **C)** *c4-2*. Root skewing angles were measured and a data was analyzed using ANOVA. Average root skewing angles and standard error are shown in Table 21. Same letter denotes no significant difference ($p>0.1$) when comparing plus and minus propyzamide treatment at a particular NaCl treatment in each genotype. A different letter denotes a significant difference when comparing plus and minus propyzamide treatment at a particular NaCl treatment in each genotype ($p\leq0.01$).

Table 21. Average root skewing angle in the presence of NaCl and propyzamide. Negative values indicate left skewing; positive values indicate right skewing. * indicates values significantly different ($p<0.01$) when comparing medium with or without propyzamide at a particular NaCl concentration in each genotype. SE = standard error.

NaCl (mM)	3 μM Propyzamide	Root Angle		
		WT	*c4-1*	*c4-2*
0	No	-16.6°	-13.3°	-10.1°
	Yes	-59.5° *	-71.2° *	-66.3° *
50	No	-3.0°	36.0°	35.3°
	Yes	0.3°	24.9°	16.3°
SE		3.5°	3.2°	3.6°

type and the *a1-1* mutant (gift from Dr. Alison DeLong) were grown on Basic Medium. After stratification, the plates were placed vertically at 25°C with a 16-hr photoperiod. After three days, healthy seedlings were transferred to Basic Medium with or without 75 mM supplemental NaCl and grown for another 7 days. When grown in Basic Medium only, roots of wild type displayed slight left skewing as observed before (Figures 30 and 40A). *a1-1* mutants also displayed slight left skewing on Basic Medium (Figure 40A). When supplemental 75mM NaCl was added to the medium, roots of wild type displayed slight right skewing as expected (Figure 40B). The roots of *a1-1* mutants exhibited a root curling phenotype on medium with 75 mM NaCl (Figure 40B). The PP2A A1 subunit is already known to be involved in auxin transport (Garbers et al., 1996; Rashotte et al., 2001) and the *a1-1* mutant also displayed an altered root direction growth phenotype on medium with supplemental NaCl. Since both PP2A A1 subunit and C4 subunit mutants showed skewing or curling phenotypes in the presence of NaCl, these results suggest a general role for PP2A in regulating auxin transport.

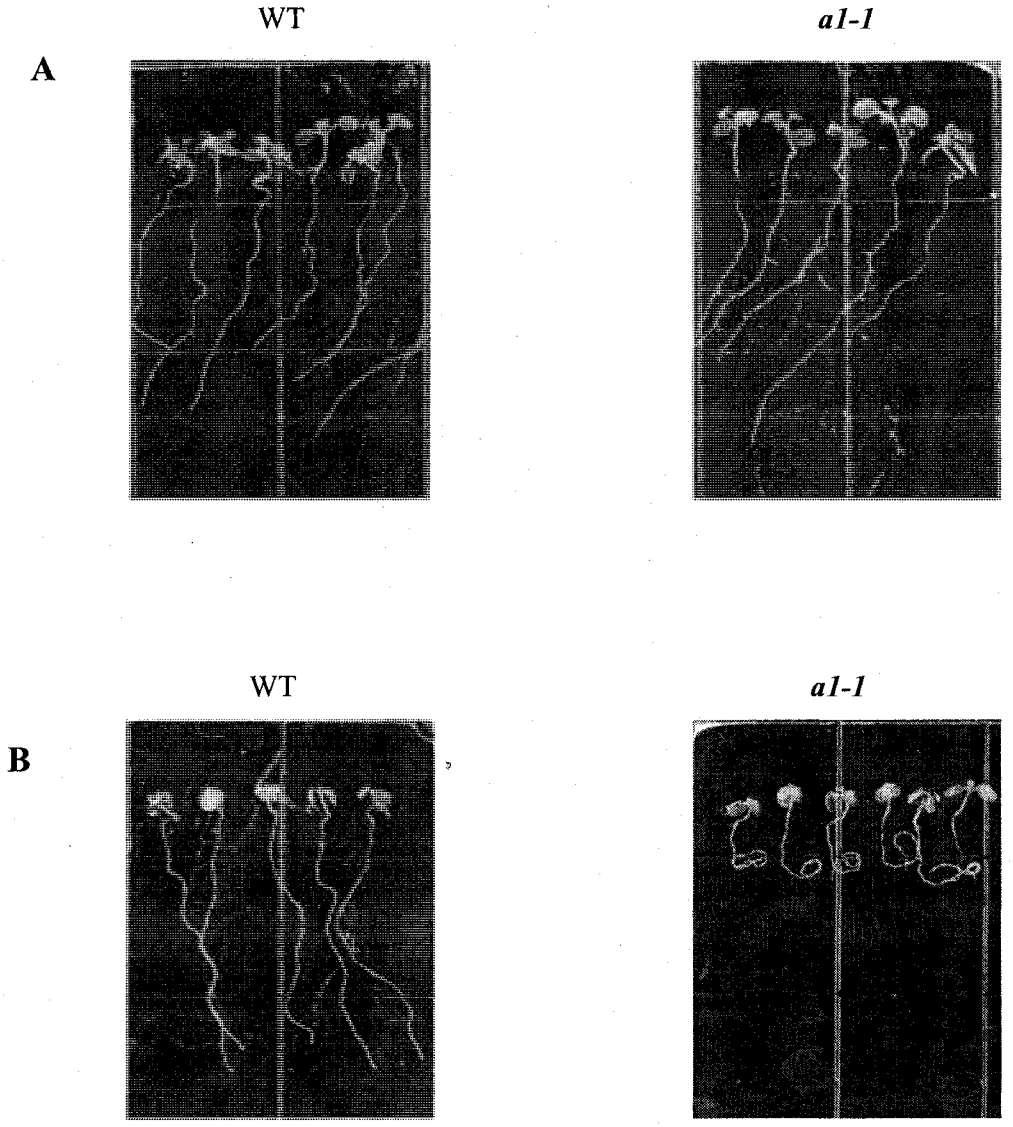


Figure 40. Root curling phenotype of wild type (WT) and PP2A *a1-1* mutant seedlings in the presence of 75 mM NaCl. **A.** no supplemental NaCl **B.** 75 mM supplemental NaCl

Discussion

Protein phosphorylation is involved in many cellular activities in eukaryotic cells (Cohen, 2001). Compared with kinases, which have been intensively studied, protein phosphatases have received less attention even though elucidating the function of phosphatases is equally as important as studying kinases. Pharmacological evidence has indicated the involvement of PP2A in different physiological processes in plants (Huber et al., 1992; Li et al., 1994; MacKintosh et al., 1994; Monroy and Dhindsa, 1995; Foissner et al., 2002). However, a genetic approach has only revealed a function for three specific PP2A subunits and most of these functions are related to hormone signaling. PP2A A1 is involved in auxin, ABA and ethylene signaling (Garbers et al., 1996; Kwak et al., 2002; Larsen and Cancel 2003; Michniewicz et al., 2007). The *TON2* gene encoding a putative PP2A B″ subunit affects the microtubules in the cortical cytoskeleton and a mutation causes dwarf plants with abnormally shaped cells (Camilleri et al., 2002). The C2 catalytic subunit of PP2A has been identified as a negative regulator of the ABA pathway (Pernas et al., 2007).

In this study, reverse genetics was used to address the function of protein phosphatase 2A C subunits in *Arabidopsis*. I screened for phenotypes under different hormone, developmental and nutrient treatments. In most of the screens, PP2A C subunit mutants did not show any differences from wild type. However when PP2A *c4* mutants were grown in the presence of NaCl, roots of PP2A *c4* mutants showed a strong right

skewing when viewed from the top of the plates. This is the first report of a function for the PP2A C4 subunit in *Arabidopsis*.

The exact mechanism for root skewing remains unclear. A summary of the current model based on genetic, physiological and microscopy data as reviewed in Oliva and Dunand (2007) is shown in Figure 41. Root skewing is likely induced by both touch stimuli and gravity responses. Following the perception of the stimulus, subsequent events can be thought of as two pathways. In the first pathway, root skewing is initiated by rotation of the root cell files. The important events in the first pathway are summarized on the left side of Figure 41. Straight growth of roots requires transverse arrays of cortical microtubules in the cells in the elongation zone so that cell expansion can occur along the cells' longitudinal axis. After perceiving stimuli, the cortical cytoskeleton shifts from transverse arrays to helical assays, causing transverse deposition of cellulose microfibrils in the cell wall, thereby resulting in cell expansion at an angle to the longitudinal axis. This unequal expansion leads to cell file rotation (CFR) with the final consequence of root skewing. In the second pathway, unequal cell elongation mediated by auxin leads to skewing and is listed in the right part of Figure 41. Unequal auxin concentration across the root elongation zone results in differential cell elongation across the root. The cells exposed to more auxin elongate less than the cells that perceive lower auxin concentration. More elongation of one side results in root skewing.

One way to understand the mechanism for root skewing is to identify the mutants that are affected in this process. The most common way to screen for root skewing

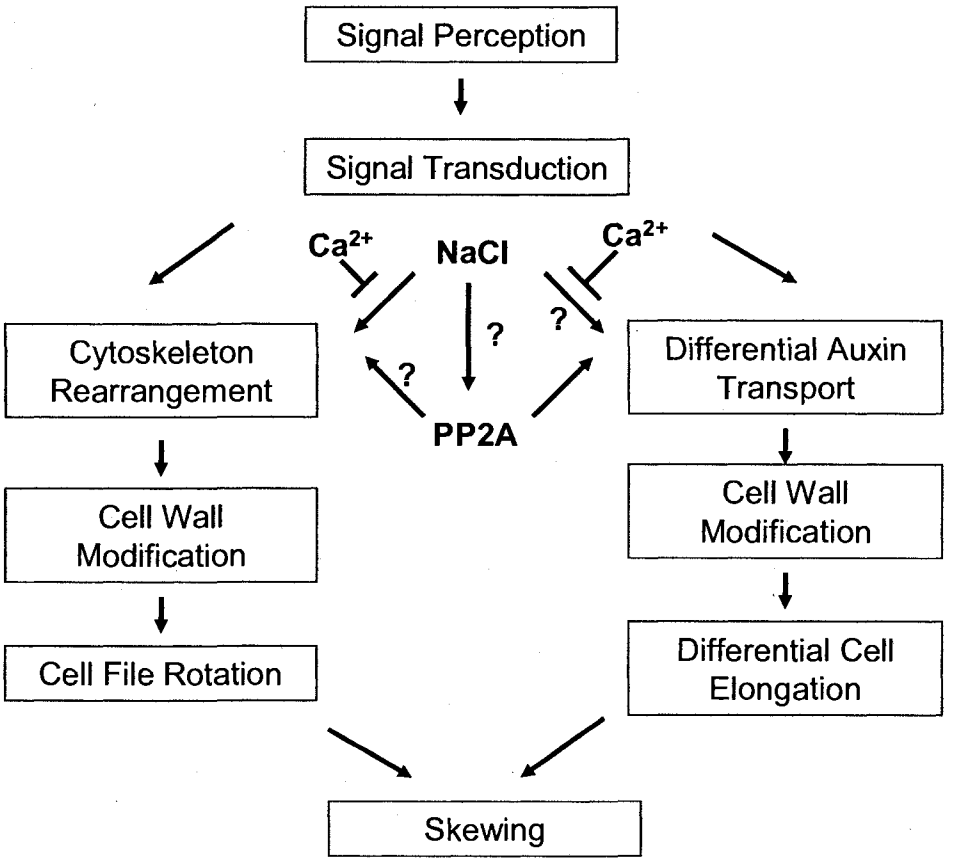


Figure 41. Simplified model of root skewing. Supporting data for this model is described in the text. Question marks indicate hypothetical interactions that may occur, based on data reported in this thesis.

mutants is to grow seedlings on plates containing 1.5% agar and tilted backward 45°. Roots cannot penetrate the growth medium at this agar concentration. When grown on the tilted plates, the wild type root tip, which is rotating clockwise as it elongates, continuously hits the agar surface and changes direction causing the root deviate from a strict gravitational direction (Migliaccio and Piconese, 2001). Mutants have been identified that display exaggerated skewing compared with wild type and the direction of skewing can be either leftward or rightward when viewed from above the plates. Left skewing mutants include *sku1*, *sku2*, *lefty1*, and *lefty2* (Rutherford and Masson, 1996; Thitamadee et al., 2002). Right skewing mutants include *spr1*, *spr2* and *wvd2-1* (Furutani et al., 2000, Yuen et al., 2003). The products of some of these genes have been identified and shown to be proteins associated with the cytoskeleton. For example, LEFTY1 and LEFTY2 are different α-tubulin isoforms (Thitamadee et al., 2002). The SPR1 protein is a microtubule interacting protein that is preferentially localized to the plus ends of cortical microtubules (Sedbrook et al., 2004). SPR2 is also a microtubule-associated protein (Furutani et al., 2000; Buschmann et al., 2004). The functions of the proteins encoded by other genes such as *SKU1* and *SKU2* are still unknown.

Researchers seeking to understand the basis for root skewing have noticed that the normally straight epidermal cell files observed in wild type seedlings are often altered in left-skewing mutants, appearing as epidermal cell files with a left-handed rotation while right-skewing mutants do the opposite (Rutherford and Masson, 1996; Furutani et al., 2000; Thitamadee et al., 2002; Yuen et al., 2003, Ishida et al., 2007). This observation suggested that the cell wall, whose direction of expansion is determined by the

orientation of cellulose fibrils in the wall, is being affected by changes in the cortical cytoskeleton and resulting in skewing (Rutherford and Masson, 1996; Furutani et al., 2000; Thitamadee et al., 2002; Yuen et al., 2003; Ishida et al., 2007; Oliva and Dunand, 2007). Involvement of the cortical cytoskeleton in root skewing is supported by observing roots following application of MT-depolymerizing drugs such as propyzamide and oryzalin. In both wild type and root skewing mutants, microtubule depolymerizing drugs changed the angle of root skewing and this was correlated with rearrangement of the MTs, leading to modified cell wall expansion and a change in CFR (Rutherford and Masson, 1996; Furutani et al., 2000; Thitamadee et al., 2002; Yuen et al., 2003; Sedbrook, et al., 2004; Oliva and Dunand, 2007). These data indicate a potential role for the cytoskeleton in root skewing (Figure 41, left). Although root skewing is associated with CFR, there is at least one report that CFR is not necessary for skewing (Buer et al., 2003). The PP2A *c4* mutant studied here is different from previously described skewing mutants in two ways. First, the *c4* mutants displayed skewing when grown on vertically-oriented plates containing 1% agar and the plates did not have to be tilted backward. Second, the *c4* mutants exhibited root skewing only in the presence of NaCl but were indistinguishable from wild type on Basic Medium. So, NaCl functions as a signal to induce the root skewing in *c4* mutants.

My result showed that roots of PP2A *c4* mutants displayed a strong right skewing and decrease of root elongation in the presence of NaCl. Recent data also shows a connection between PP2A and salt treatment. Root elongation of mutation in PP2A A subunit (*rcn1*) seedlings decreased more than wild type in the presence of NaCl and KCl indicating that RCN1 is required for normal stress tolerance in seedling roots (Blakeslee

et al., 2008), this result supports my hypothesis that PP2A plays a critical role in responding to NaCl treatment.

Previous evidence showed that NaCl can affect the organization of microtubules and alter the direction of root growth (Figure 41, left). In tobacco By-2 cells, cortical MT arrays reorganized from a transverse orientation to a more random arrangement after treatment with 150 mM NaCl (Dhonukshe et al., 2003). There is evidence that reorganization of microtubules in the presence of NaCl may be mediated by the salt overly sensitive (SOS) signal transduction pathway (Shoji et al., 2006). Mutations in *SOS1* and *SOS2* suppressed both cortical microtubule disruption and helical growth of the *spiral1* mutant. Also, *sos1* and *sos2* mutants displayed abnormal responses to the microtubule-interacting drugs taxol and oryzalin (Shoji et al., 2006). A recent paper indicated that depolymerization and reorganization of cortical microtubules were critical for the plants' ability to withstand salt stress (Wang et al., 2007). In the presence of NaCl, wild type *Arabidopsis* seedlings displayed right-handed root skewing and underwent transient depolymerization of the cortical microtubules (Wang et al., 2007). Stabilization of microtubules with paclitaxel resulted in increased seedling death from salt stress while depolymerization of microtubules with oryzalin or propyzamide prevented seedling death (Wang et al., 2007). All these data provide a connection between microtubules and NaCl stress.

Phosphorylation is known to affect the stability of the plant cytoskeleton. Chemicals that inhibit either kinases or phosphatases caused disorganization of cortical microtubules (Baskin and Wilson, 1997; Figure 41, left). NaCl was not required for this disorganization indicating that protein phosphorylation/dephosphorylation alone can alter

cytoskeleton integrity. In the current study, when PP2A *c4* mutants were grown in the presence of NaCl, the roots showed a strong right skewing when viewed from the top of the plates. This result demonstrates a connection between NaCl, root skewing and PP2A for the first time. My discovery that the A1 subunit mutant *rcn1* has a root curling phenotype in the presence of NaCl provides further support for this interaction.

So far, I do not have evidence to prove that the root skewing in the *c4* mutant is caused by rearrangement of the cytoskeleton or by CFR, but my data show that NaCl and the anti-microtubule drug propyzamide have antagonistic effects on the direction of root skewing in *c4* mutants. Propyzamide induced strong left skewing in both wild type seedlings and PP2A *c4* mutants seedlings on Basic Medium while NaCl induced right skewing. When both supplemental NaCl and propyzamide were added to the medium, the roots of wild type seedlings were almost straight or showed a slight rightward skew. The *c4* mutant seedlings still skewed rightward but the skewing was less strong than the skewing observed with NaCl alone. Combined with the previous observation that cortical microtubules depolymerize then reorganize themselves when plants undergo salt stress, I hypothesize that when plants undergo NaCl treatment, PP2A plays an important role in ensuring that the cytoskeleton is in the correct orientation to maintain proper gravitropic growth direction. In *c4* mutants, the repolymerization may not occur in the correct way leading to a strong right skewing phenotype. The MT-depolymerizing drug propyzamide may affect the rearrangement of the cytoskeleton in *c4* mutants leading to a reduced right skewing phenotype. Additional experiments to observe cortical microtubules in root cells of *c4* mutants will provide more evidence to help us understand how the PP2A C4 subunit is involved in this process (see Chapter V).

The rearrangement of the cytoskeleton is only one aspect of root skewing (Figure 41, left); differential auxin transport may also be involved (Figure 41, right). There is an established connection between PP2A and auxin transport. The PP2A A subunit mutant *rcn1* was identified based on an altered root growth pattern in the presence of an auxin transport inhibitor (NPA) which normally causes roots to fail to curl when they contact the bottom of a plate (Garbers et al., 1996). PIN proteins are auxin transport efflux carriers located in the plasma membrane (Paponov et al., 2005). The *Arabidopsis* PIN family consists of eight members. While not all PINs have been characterized yet, those that have are expressed in different root cells and often localize at different sides of the cells (Paponov et al., 2005). For example, PIN1 localizes at the basal side of the vascular cells, PIN2 localizes at the apical side of the lateral root cap and epidermal cells but at the basal end of cortical cells, and PIN4 localizes at the basal side of the central root meristem cells. The polar targeting of PIN proteins plays an important role in determining the direction of auxin transport (Feraru and Friml, 2008). PIN1, PIN2 and PIN4 are phosphorylated by PINOID kinase and may be dephosphorylated by PP2A (Friml et al., 2004; Michniewicz et al., 2007). PINOID kinase and PP2A phosphatases appear to act antagonistically on PIN proteins determining the apical-basal targeting of these proteins and leading to the correct auxin distribution in the root (Michniewicz et al., 2007). Asymmetric distribution of PIN proteins within root cells causes asymmetric distribution of auxin in roots, resulting in gravitropic root response (Friml and Palme, 2002).

A recent paper showed that NaCl modulates root growth direction by reducing the gravity response (Sun et al., 2008), establishing a connection between auxin transport and

salt. Amyloplasts in the root cap columella cells function in sensing gravity signals. Amyloplasts in the *Arabidopsis* root cap columella cells were rapidly degraded when seedlings were exposed to NaCl. Degradation of amyloplasts led to the reduction of the gravity response (Sun et al., 2008). NaCl-induced amyloplasts degradation was correlated to the distribution of PIN2. It is likely that NaCl induces mislocation of PIN2 resulting in improper auxin distribution in the root elongation zone although this was not shown. Asymmetric auxin distribution would cause unequal cell elongation between two sides of roots with a final consequence of change of root growth direction.

My results showed that auxin transport may be involved in root skewing in *c4* mutants in the presence of NaCl by using the non-competitive auxin transport inhibitor TIBA. When 50 mM supplemental NaCl was added to the Basic Medium, the roots of *c4* mutant seedlings showed a stronger right skewing than wild type, but on medium with 50 mM NaCl and 1 μM TIBA, the roots of *c4* mutant plants exhibited a root curling phenotype (Figure 38). This study demonstrated a connection between PP2A, NaCl and auxin transport. Combined with previous evidence that PP2A affects the localization of PIN proteins and that salt treatment altered the distribution of PIN2, I hypothesize that NaCl may induce dephosphorylation of one or more PIN proteins catalyzed by PP2A. In *c4* mutants, dephosphorylation is inhibited, resulting in changes of abundance and distribution of PINs, leading to differential auxin transport. This intracellular redistribution of auxin could result in cell wall modification and differential cell elongation with the final consequence being a change in the direction of root growth (Figure 41, right). This model predicts that factors inhibiting auxin transport should

enhance root skewing by magnifying the effect of mislocalized PIN proteins. My results support this model because TIBA and NaCl together changed root growth direction of *c4* mutants more than NaCl alone.

My results indicate that Ca^{2+} affects the root skewing phenotype caused by NaCl by decreasing the degree of right skewing. Both wild type and mutant seedlings exhibited similar trends in root growth in the presence of NaCl, whether or not $CaCl_2$ was added. In addition, supplemental $CaCl_2$ decreased the magnitude of the rightward root skewing phenotype caused by NaCl in both wild type and PP2A mutants. The decreased magnitude of the rightward root skewing was greater in PP2A mutants than wild type. I hypothesize that Ca^{2+} may decrease rightward root skewing in the presence of NaCl in several ways. First, extracellular Ca^{2+} inhibits the initial Na^+ influx into cells and the initial influx step is a key determinant of overall shoot Na^+ accumulation (Essah et al., 2003). Thus, the increased extracellular Ca^{2+} concentration in my experiment likely inhibited NaCl influx, resulting in less NaCl stress and decreased root skewing angles. Second, wild type seedlings displayed depolymerization and reorganization of microtubules under NaCl stress accompanying increased intracellular Ca^{2+} concentration (Wang et al., 2007). NaCl activates calcium channels, leading to increased intracellular Ca^{2+} concentration (Trion et al., 1998). An increased intracellular Ca^{2+} concentration may be related to reorganization of the cortical microtubules and seedling survival (Wang et al., 2007). In my study, the magnitude of the rightward root skewing phenotype decreased when supplemental $CaCl_2$ was added. I hypothesize that adding supplemental $CaCl_2$ may increase the intracellular Ca^{2+} concentration leading to reorganization of

microtubules with a final consequence of decreased root skewing. Third, calcium is reported to enhance auxin transport in maize roots (Lee and Evans 1985). In horizontally oriented roots, movement of labeled IAA in both direction across the elongation zone was 3-fold faster in the presence of $CaCl_2$ than in the absence of $CaCl_2$ (Lee and Evans, 1985). I hypothesize that $CaCl_2$ may increase auxin transport in the *c4* mutant, leading to a decreased auxin gradient across the roots, thereby decreasing the rightward root skewing phenotype. It is still uncertain how Ca^{2+} affects root skewing in my study, but the skewing angles did decrease when extra Ca^{2+} was present in the medium indicating that Ca^{2+} plays an important role in regulating root growth in the presence of NaCl (Figure 41).

PP2A is a heterotrimeric enzyme and in theory, there are 255 possible different heterotrimers that might exist in *Arabidopsis* considering the number of genes that have been identified for each of the three subunits. My results indicate that, among these C subunit mutants that I identified, only *c4* mutants showed a deviation of root angle in the presence of NaCl even though all C subunits are highly conserved at the amino acid level. I can not conclude that the root skewing phenotype is specific to the *c4* mutant because I only have confirmed knockout mutants for the C1, C3 and C4 subunits. However, the five PP2A C subunit isoforms are divided into two subgroups by amino acid sequence similarity. C1, C2 and C5 belong to one group and are 89% - 93% identical to each other, while C3 and C4 form the second group and are 98% identical to each other (Figures 1 and 27). Interestingly, even though C3 and C4 belong to the same subgroup based on amino acid similarity, *c3* knockout mutants did not show the root skewing phenotype. This result suggests nonequivalent roles of these PP2A C subunit isoforms. Different roles for PP2A subunits have also been reported for the A subunit. Three genes encode

PP2A A subunits in *Arabidopsis* and they also share highly conserved amino acid sequences (Figure 2). However, the A1 subunit plays the predominant role among the three subunits since single *a2* or *a3* mutants have no discernable phenotype and only double mutants with *a1* show severe phenotypes (Zhou et al., 2004). Mammalian A1 and A2 isoforms show striking differences in their B and C subunit binding characteristics and expression levels in normal tissues (Zhou et al., 2003). My data and the data from the A subunit mutants indicate that PP2A heterotrimers in *Arabidopsis* may show similar specialization.

Overall, I demonstrated that PP2A may be involved in several pathways affecting root growth in the presence of NaCl (Figure 41). The interconnection between these pathways is not understood but the PP2A *c4* mutant may provide insight into the connection between them. Among the genes and mutants identified so far (Oliva and Dunand, 2007), no protein phosphatase has been reported to take part in root skewing. The findings from this study have provided evidence of a new frontier for PP2A research in plants.

CHAPTER V

FUTURE DIRECTIONS

Summarizing all of the data in Chapters II, III and IV, 13 homozygous mutant lines generated by the insertion of foreign DNA (T-DNA) in the five *Arabidopsis* PP2A C subunit genes were identified by PCR. No full-length mRNA was produced in five of these 13 homozygous mutant lines as determined by RT-PCR. At 50 mM or greater concentration NaCl, roots of *c4* mutant seedlings grown on vertically-oriented plates had a strong right-skewing growth pattern while wild type showed only a slight right skewing. Several future directions are listed below that will enhance our knowledge of function of PP2A in plants.

Establishment of *a1c4* Double Mutants

My results suggest that the PP2A C4 subunit might play an important role in root growth in the presence of NaCl. Furthermore, mutants in the PP2A A1 subunit showed root curling in vertically-oriented plates in the presence of NaCl. These data suggest that PP2A might play an important role in root growth in the presence of NaCl. Therefore, it could be informative to make PP2A double mutants or higher order mutants. The first double mutant that should be made is *a1c4*. To make the double mutants, heterozygotes generated by crossing will be allowed to set seed and the progeny will be screened to

identify plants homozygous for both T-DNAs by PCR. *a1c4* double mutants will be characterized for their root skewing phenotype on medium with supplemental NaCl.

Cortical Microtubule Observations

The results of the present study provide the first evidence that protein phosphatase 2A is involved in plant root growth in the presence of NaCl. A recent report showed that roots from wild type plants grown on tilted plates exhibited a skewing phenotype in the presence of NaCl and indicated that cortical microtubules must go through a depolymerization and reorganization process in order for plants to deal with salt stress (Wang et al., 2007). The results of my study have shown that both wild type and *c4* mutant seedlings responded similarly but to different degrees to the microtubule-depolymerizing drug propyzamide in the presence of NaCl. Cortical microtubule observations in root cells will provide direct evidence to help us to understand how the PP2A C4 subunit is involved in this process. The cortical microtubules in root cells can be observed by immunofluorescence microscopy, according to a protocol described by Sugimoto et al. (2000). To investigate salt stress-induced organization of the cortical microtubules, a root skewing assay in the presence and absence of NaCl treatment will be conducted. At the end of the assay period, seedling roots will be fixed with 4% (v/v) paraformaldehyde and 0.1% (v/v) glutaraldehyde and immunolabeled with a primary monoclonal antibody against β-tubulin from mouse. The samples will then be immunolabeled with a fluorescently-labeled secondary antibody. Immunofluorescence images will be collected with a confocal laser scanning microscope.

To investigate the organization of the cortical microtubules during treatment of roots with microtubule – targeting drugs, Ca^{2+} ions and auxin, seeds will be sown on the NaCl medium containing appropriate chemicals. The cortical microtubules in root cells will be observed using confocal microscopy as described above.

Detection of C Subunit Proteins

Although the size of an RT-PCR product can indicate whether enough transcript is present that a full length protein could be produced, this method does not reveal what the product of translation is. More definitive evidence can be obtained through detection of protein levels. The alignment of amino acid sequences of *Arabidopsis* PP2A C subunits shows a high level of similarity (Figures 1 and 27). Unfortunately, all five C subunits also have similar molecular weights and isoelectric points making these proteins difficult to separate by electrophoretic methods. As a result, C subunit proteins are usually visualized as a single band on a western blot (Zhou, et al., 2004). However, a noticeable difference between mutant and wild type protein patterns could likely be detected if one of the subunits is significantly more abundant than the others. This has been observed with the *A1* subunit mutants (Zhou, et al., 2004). Western blotting will be used to investigate protein expression levels in the knockout mutant plants, especially *c4-1* and *c4-2*. If the C4 proteins are fairly abundant relative to the other four C subunit proteins, then immunoblotting may help us understand the relative contribution of this single C subunit to the total amount of C subunits and will complement the data obtained from RT-PCR.

Phosphatase Activity Assay

Whether T-DNA decreases the overall activity of PP2A in plants can be investigated by measuring the total protein phosphatase activity in mutant plants. The PP2A *a1* mutant has reduced PP2A phosphatase activity compared to wild type plants indicating that the phenotype is likely due to an enzyme deficiency (Deruere et al., 1999). The impact of PP2A C subunit mutations on phosphatase activity will be determined using an in vitro phosphatase assay. The Non-Radioactive Phosphatase Assay Kit (Promega, Madison, WI) will be used to determine the amount of free phosphate generated by wild type and mutant plant extracts. The kit contains the chemically synthesized phosphopetide substrate RRA(pT)VA that is compatible with PP2A, PP2B and PP2C but not PP1. Since there is no PP2B gene in *Arabidopsis* (Luan, 2003), PP2A and PP2C can be differentiated by including different protein phosphatase inhibitors in the reaction: EGTA for PP2C and okadaic acid for PP2A. These approaches can distinguish PP2A from other protein phosphatases and, if a reduction of phosphatase activity is observed, will indicate that this reduction is from PP2A.

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