# PROTEIN PHOSPHATASE 2A CONTROLS SEEDLING GROWTH THROUGH DIFFERENTIAL REGULATION OF ETHYLENE BIOSYNTHESIS

BY

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# CHAPTER 1: INTRODUCTION

Protein phosphorylation is one of the most prevalent posttranslational modifications and has been shown to regulate myriad cellular functions in both eukaryotes and prokaryotes (Hunter, 1995; Manning et al., 2002; Wang, 2007; Moorhead et al., 2009). Protein phosphorylation is catalyzed by protein kinases, which can add a phosphoryl group to the side chain of nine amino acids including serine, threonine, tyrosine, histidine, aspartate, cysteine, arginine, lysine, and glutamate (Moorhead et al., 2009). In multicellular eukaryotes, protein kinases most commonly catalyze the addition of a phosphoryl group to an hydroxylated amino acid such as serine, threonine, and tyrosine (Moorhead et al., 2009). In humans for example, the proportion of phosphorylation on serine, threonine, and tyrosine residues are approximately 86%, 12%, and 2%, respectively (Olsen et al., 2006). In contrast, serine phosphorylation is less dominant in prokaryotes, which rely on so-called two-component histidine and aspartate phosphorelays to transduce many signaling events (Kannan et al., 2007; Macek et al., 2008; Schaller, 2008; Schaller et al., 2011). The process of phosphorylation is made reversible by the action of protein phosphatases, which oppose the action of protein kinases by catalyzing the removal of phosphoryl groups from previously phosphorylated amino acids.

**Regulation Through Phosphorylation**

Reversible protein phosphorylation can regulate protein-protein interactions, protein localization, gene transcription, enzyme kinetics, cellular signaling, and the cell cycle (Johnson and Lewis, 2001; Sontag, 2001). The addition and

removal of a phosphoryl moiety can have profound consequences for the modified protein. At physiological pH, the phosphoryl group is likely to be dianionic, imparting a double negative charge that can alter charge-based interactions in both an intra- and inter-peptide manner. Phospho-specific structural studies have indicated that two major charge-neutralizing interactions are favored by phospho-residues, specifically, interaction with main-chain nitrogens at the beginning of an alpha helix and with the side group of arginine residues (Johnson and Lewis, 2001). In addition to charge effects, the phosphoryl group is relatively bulky and can also cause steric hindrance.

The importance of protein phosphorylation is highlighted by the fact that there are 62 genes encoding two-component signaling phosphorelays in *E. coli*, representing 1.5% of the genome (Johnson and Lewis, 2001). Although His-Asp-dependent two-component relays have been considered the predominant bacterial phosphorylation machinery, recent analysis of the microbial kinome as part of the Oceanic Metagenomics project has indicated that bacterial genomes may encode more eukaryotic protein kinase-like genes (which phosphorylate serine, threonine, and tyrosine residues) than two-component phosphorelay genes (Kannan et al., 2007). Although there are fewer examples of two-component signaling in eukaryotes, plants use two-component-like kinases for the perception of two major hormones, ethylene and cytokinin (Schaller, 2008; Schaller et al., 2011). As in prokaryotes, the importance of phosphorylation is reflected in eukaryotic (yeast and mammalian) genomes where between 1.5 –

2.5% of all genes encode protein kinases (Manning et al., 2002). Interestingly, the vast majority of eukaryotic protein kinases evolved from a common ancestor and belong to a single superfamily of eukaryotic protein kinases (Manning et al., 2002).

Protein kinases generally consist of a catalytic subunit that is juxtaposed to a regulatory domain, creating an enzyme with relatively high intrinsic substrate specificity (reviewed in Wang, 2007). In addition to intramolecular substrate specificity determinants, protein kinases may also interact with a range of activators, inhibitors, and scaffolding proteins to provide additional regulation of activation and substrate specificity (Johnson and Lewis, 2001). Eukaryotic genomes encode a vast array of protein kinase catalytic subunits. Arabidopsis is no exception, with genes encoding over 1000 typical protein kinases present in the genome, representing approximately 4% of the total genes (Wang, 2007). The relative increase in kinase encoding genes in plants compared to other eukaryotes (~4% versus ~2%) may be due to the challenges of regulating the expanded metabolic repertoire, the need for a rapid and flexible defense network, or the response to abiotic stress conditions associated with a sessile lifestyle. Consistent with this notion, the vast majority (606) of Arabidopsis kinases belong to the receptor-like protein kinase (RLK) family. The Arabidopsis genome also encodes 84 members of the calcium-dependent protein kinase (CDPK)-SNF1-related kinase (SnRK) superfamily, 90 mitogen-activated protein kinase (MAPK) cascade members, and 17 histidine kinase-like proteins resembling bacterial two-

component signaling proteins (MAPK Group, 2002; Hrabak et al., 2003; Schaller, 2008). In contrast to the human genome, which encodes over 100 protein tyrosine kinases (PTKs), there are no PTKs encoded in the Arabidopsis genome (Moorhead et al., 2009).

**Eukaryotic Protein Phosphatases**

In sharp contrast to the vast number of protein kinases encoded in the Arabidopsis genome, there are only 112 genes encoding protein phosphatase catalytic subunits, roughly one tenth the number of protein kinases (Kerk et al., 2002; Moorhead et al., 2009). Additionally, protein phosphatases have evolved separately several times (Kerk et al., 2002; Moorhead et al., 2009). Regulation of phosphatase specificity reflects this evolutionary history. The members of two protein phosphatase families (PTP and PPM) contain both a catalytic and regulatory domain in the same polypeptide and, similarly to eukaryotic protein kinases, have expanded catalytic subunit number to ensure substrate specificity. In contrast, the two predominant serine/threonine phosphatases, PP1 and PP2A feature very few catalytic subunits and instead rely on many interactions with diverse regulatory subunits to generate a plethora of unique phosphatases all containing the same catalytic subunit.

Protein phosphatases can be broken down into three major categories based on catalytic action; serine/threonine phosphatases (STPs) act on serine and threonine residues, protein tyrosine phosphatases (PTPs) dephosphorylate

phospho-tyrosine residues specifically, and dual specificity phosphatases (DSPs) dephosphorylate serine, threonine, and tyrosine residues. In agreement with the absence of tyrosine protein kinases in the Arabidopsis genome, there is also a lack of classical protein tyrosine phosphatases (Moorhead et al., 2009). Surprisingly, the proportion of serine, threonine, and tyrosine phosphorylation in Arabidopsis is approximately 85%, 11%, and 4%, respectively, which closely matches the human phosphorylation profile (Sugiyama et al., 2008). This suggests that nearly all tyrosine phosphorylation in Arabidopsis is regulated by dual-specificity protein kinases and phosphatases.

The serine/threonine phosphatases are further divided into two families based on their metal dependence: the phosphoprotein phosphatase (PPP) family and the metallo-dependent phosphoprotein phosphatase (PPM) family. PPP family members PP1, PP2A, PP2B (aka PP3 or Calcineurin), PP4, PP5, PP6, PP7, and PPP Kelch phosphatases, do not require exogenous metal ions for activity (in protein extracts). The PPM family consists of PP2Cs, which require $Mg^{2+}$ for activity (reviewed in Farkas et al., 2007).

PP2Cs are monomeric protein phosphatases that are common to all eukaryotes and reliant on $Mg^{2+}$ for catalytic activity (reviewed in Wang, 2007). Compared with those of other eukaryotes, the plant PP2C family has been greatly expanded, with approximately 69 PP2Cs encoded in the Arabidopsis genome. N- or C-terminal extensions from the PP2C catalytic domain are thought to

provide substrate specificity (Schweighofer et al., 2004). PP2Cs in plants and PTPs in mammals are similar in that both families of phosphatases have used amplification of catalytic subunit numbers to expand substrate specificity. This mirrors the mechanism through which eukaryotic protein kinases achieve substrate specificity. Indeed, in mammals, there are similar numbers of tyrosine kinases and tyrosine phosphatases, with approximately 100 members each (Bollen et al., 2010).

PP2Bs function as heterotrimeric enzyme complexes containing the catalytic calcineurin A (CNA) subunit, the regulatory calcineurin B (CNB) subunit and calmodulin (Roy and Cyert, 2009). In the absence of calcium signaling, the autoinhibitory domain of CNA inactivates the catalytic domain. In the presence of cytosolic $Ca^{2+}$, the binding of both calcium and calmodulin releases the autoinhibitory domain and allows phosphatase activity (Roy and Cyert, 2009). Surprisingly, no PP2B catalytic subunits have been found in higher plants (Farkas et al., 2007).

In contrast, the PP7 and PPP Kelch families appear to be specific to plants (Farkas et al., 2007). Like calcineurin, PP7 is regulated by calcium and calmodulin. However in the case of PP7, calmodulin binding is reported to inhibit phosphatase activity (Andreeva et al., 2001). PP7 has been implicated in cryptochrome-mediated blue light signaling, since silencing PP7 results in the loss of both blue light-induced inhibition of hypocotyl elongation and blue light

regulated gene transcription (Moller et al., 2003; Ohgishi et al., 2004). The four PPP Kelch phosphatases in Arabidopsis (BSU1 and BSL1, 2, and 3) are distantly related to PP1 and are involved in regulating brassinosteroid signaling (Mora-Garcia et al., 2004).

Although the total cellular serine/threonine phosphatase activity incorporates the activities of all PPP and PPM phosphatases, just two members of the PPP family, PP1 and PP2A, constitute up to 85% of the cellular serine/threonine phosphatase activity (MacKintosh and Cohen, 1989). As such, both PP1 and PP2A regulate myriad cellular functions. Interestingly, while the Arabidopsis genome encodes over 1000 serine/threonine protein kinases, there are only 9 PP1 catalytic subunits and 5 PP2A catalytic subunits (Farkas et al., 2007). Instead of large numbers of catalytic subunits, PP1 and PP2A rely on interactions with diverse regulatory proteins to direct subcellular localization and substrate specificity. For example, PP1 interacts with one or two regulatory proteins at a time to direct substrate specificity and in mammals nearly 200 PP1 interacting proteins have been identified (Bollen et al., 2010). Like protein kinases, the number of genes encoding protein phosphatase catalytic subunits has expanded in plants (Moorhead et al., 2009).

Analysis of the *in vivo* functions and regulatory proteins associated with PP1 in plants has only recently been initiated (Takemiya et al., 2009; Templeton et al., 2011). In contrast, many PP2A regulatory subunits and processes regulated by

PP2A have been identified in Arabidopsis (DeLong, 2006). PP2A is a highly abundant phosphatase, constituting up to 1% of total cellular protein and accounting for up to half of all serine/threonine protein phosphatase activity (Ruediger et al., 1991; Lin et al., 1998). PP2A catalytic subunits employ the use of two additional regulatory proteins for targeting specificity and in this manner are typical of the combinatorial targeting mechanism employed by many eukaryotic phosphatases (Figure 1).

In contrast to eukaryotic protein kinases, which are derived from a common ancestor, protein phosphatases exhibit convergent evolution, as multiple divergent families of protein phosphatases have adopted a similar catalytic pocket (Manning et al., 2002). This most likely accounts for the differences in regulatory approaches used by PPM and PTP (many catalytic subunits) versus PP1 and PP2A (many regulatory subunits) phosphatases. In addition, this has allowed the identification of separate phosphatase activities based on subtle differences in enzymatic properties. The activities of serine/threonine protein phosphatases were originally characterized based on their metal ion dependence and sensitivity to various inhibitors (MacKintosh et al., 1990; reviewed in (Hunter, 1995); see Table 2). PP2C and PPM phosphatase activities are easily separated from those of PP1 and PP2A because of their requirement for $Ca^{2+}$ and $Mg^{2+}$ ions, respectively. A defining quality of PP1 is inhibition by two small peptide inhibitors, termed inhibitor 1 and 2 (I1, I2), whereas PP2A is insensitive. Additionally, PP2A is more sensitive to a class of small molecule inhibitors that

include okadaic acid (OA) and cantharidin (CT). PP2A is fully inhibited by 1nM OA and PP1 by 1000nM OA, with $IC_{50}$ values of 0.1 and 10nM, respectively (MacKintosh et al., 1990; see Table 2). Interestingly, an explanation for this discrepancy is suggested by the crystal structures of the PP1 and PP2A catalytic subunits. Although the catalytic subunits of PP1 and PP2A exhibit over 50% sequence identity, the 100-fold difference in sensitivity to OA could be explained by a difference in four key amino acids, Gln122, Ile123, His191, and Trp200 (all amino acid positions refer to the human PP2A sequence; (Xing et al., 2006)). These four amino acids provide a "hydrophobic cage" in PP2A that promotes interaction with the hydrophobic end of OA. These hydrophobic residues are absent in PP1, likely resulting in far weaker binding and inhibition (Xing et al., 2006).

**Protein Phosphatase 2A**

Protein phosphatase 2A is highly conserved throughout eukaryotic evolution and acts predominantly as a holoenzyme containing three subunits, a scaffolding A subunit, a catalytic C subunit, and a regulatory B subunit (Figure 1). The binding of regulatory A and B subunits to PP2A-C increases enzyme specificity and catalytic activity towards physiologically relevant substrates, while simultaneously decreasing nonspecific phosphatase activity towards non-substrates (Tung et al., 1985; Turowski et al., 1997; Price and Mumby, 2000). Although both PP2A-A and B subunits allosterically regulate PP2A-C activity, PP2A-A is conventionally referred to as the structural or scaffolding subunit and PP2A-B is referred to as

the regulatory subunit. PP2A-B subunits are diverse and group into four distinct families: B, B', B", and B"' (discussed in further detail below). The entire complement of regulatory B subunits will be referred to as 'PP2A-B', while individual families or specific subunits will be referred to with a superscript annotation such as PP2A-$B^B$.

**The PP2A Scaffolding A Subunit**

The PP2A-A subunit is a horseshoe-shaped scaffolding platform composed of 15 tandem repeats of a 39 amino acid sequence known as a HEAT (Huntingtin, Elongation factor 3, PP2A A-subunit, and TOR; Andrade and Bork, 1995) repeat (Ruediger et al., 1992; Ruediger et al., 1994; Figures 1-2). Each HEAT motif consists of two anti-parallel alpha helices, which are connected by a loop, termed the intrarepeat loop. The intrarepeat loops of PP2A-A form a contiguous ridge along one surface of the A subunit (Zhou et al., 2003; Xing et al., 2006; Xu et al., 2006). Interestingly, this ridge is the most highly conserved portion of PP2A-A, consistent with the role it plays in mediating interactions with both B and C subunits (see below). In addition to the intrarepeat loop, a series of interrepeat loops connect adjacent HEAT repeats. HEAT repeats are present in several proteins whose primary function is to promote protein-protein interactions (Andrade et al., 2001). The stacking of the anti-parallel alpha helices present in each HEAT repeat and the manner in which they are linked to each other and to neighboring repeats by flexible loops affords conformational elasticity. This structure is consistent with the function of the PP2A-A subunit as a flexible

protein-protein interaction scaffold. Interestingly, a recent report suggested that the intrinsic flexibility of the PP2A-A subunit might provide an important link between force and catalysis in addition to facilitating interactions between the PP2A-C subunit and diverse regulatory B subunits (Grinthal et al., 2010).

A well-established method for transformation of mammalian cell lines involves infection with either polyoma virus or SV40 (Ruediger et al., 1992; Ruediger et al., 1994). Part of the transforming property both of these viruses share is the alteration of PP2A activity through binding of their respective small-T antigen, which displaces normal regulatory PP2A-B subunits (reviewed in Arroyo and Hahn, 2005). Once PP2A-A and C subunits were identified as small-T interacting proteins, and thus crucial regulators of cellular transformation, one priority of PP2A research was to determine the binding sites within PP2A-A for both small-T antigens and PP2A-C. An interaction study based on sequential truncation of PP2A-A was able to grossly map the interaction domains used by PP2A-C and small-T antigens. The PP2A-C subunit required the PP2A-A C-terminus (HEAT repeats 11-15) for binding, while small-T antigens required the PP2A-A N-terminus (HEAT repeats 1-9 and 3-7 for polyoma and SV40, respectively) for binding (Ruediger et al., 1992). Site-directed mutagenesis of conserved amino acid residues within the intrarepeat loop regions of PP2A-A identified the specific loop regions required for interaction with regulatory B subunits, small-T antigens, and the catalytic subunit. PP2A-C binds to the intrarepeat loops of HEAT repeats 13, 14, and 15, while PP2A-B subunits and

both polyoma and SV40 small-T antigens require the intrarepeat loops of HEAT repeats 4, 5, and 6 (Ruediger et al., 1994). This initial work has been refined due to the recent progress that has been made in the crystallization of PP2A holoenzyme complexes.

Crystal structures of PP2A holoenzymes have shown that an extensive network of hydrogen bonds is responsible for the tight binding between PP2A-A and C and to a lesser extent PP2A-A and B (Xing et al., 2006; Xu et al., 2006; Cho and Xu, 2007; Xu et al., 2008; Figure 2). The PP2A-C subunit binds to the ridge formed by the intraloops of HEAT repeats 11 – 15 (Xing et al., 2006; Figure 2). A crystal structure of the PP2A holoenzyme containing PP2A-$B^{B'gamma1}$ shows that this regulatory subunit binds to the ridges of HEAT repeats 2 – 8 and also interacts directly with the C subunit (Xu et al., 2006; Figure 2). In contrast, the crystal structure of the PP2A holoenzyme containing PP2A-$B^{Balpha}$ shows that this regulatory subunit binds to the PP2A-A ridge formed by HEAT repeats 3 – 7 and makes fewer intermolecular bonds with the PP2A-C subunit (Xu et al., 2008; Figure 2).

**The PP2A Catalytic C Subunit**

The PP2A-C subunit contains the catalytic active site of the enzyme and exhibits high sequence and structural homology to the other members of the PPP family including PP1, PP2B, PP4, and PP5 (Mumby, 2007). These phosphatases employ a characteristic $\alpha/\beta$ fold in the active site that coordinates two catalytically

active manganese metal ions (Xing et al., 2006; Xu et al., 2006). Although PP2A requires $Mn^{2+}$ for catalytic activity, it is not classified as a metal-dependent phosphatase because the $Mn^{2+}$ ions are bound tightly in the folded protein and exogenous $Mn^{2+}$ is not required for PP2A activity in protein extracts (Table 2). In contrast to tyrosine phosphatases, which use a two-step catalytic mechanism that involves the generation of a covalent phosphoryl-enzyme intermediate, PPPs use a manganese-activated water molecule to catalyze dephosphorylation in a single step. Importantly, this precludes the use of so-called substrate-trapping mutants to identify bound substrate-enzyme intermediates (reviewed in Luan, 2003).

Several types of data also indicate that eukaryotic cells employ a mechanism that senses the amount of cellular PP2A activity and regulates the translation of new PP2A-C according to homeostatic needs. For example, although attempts to overexpress PP2A in human cell lines have resulted in the transcription of copious amounts of PP2A-C RNA, accumulation of additional PP2A-C protein is translationally repressed (Baharians and Schonthal, 1998). Conversely, cells that have been treated with okadaic acid, a highly selective phosphatase inhibitor, accumulate high levels of PP2A-C protein with low phosphatase activity (Baharians and Schonthal, 1998). These data indicate that the PP2A-C translational control mechanism functions as a readout of total PP2A activity, although the details of the mechanism are still poorly understood (Janssens and Goris, 2001).

Posttranslational modification of the C subunit also plays a prominent role in the maturation and assembly of PP2A holoenzymes. Eukaryotic genomes encode a set of three highly conserved enzymes that specifically recognize and modify PP2A-C. These include a PP2A phosphatase activator designated PTPA, as well as a dedicated methyltransferase (LCMT/PPM1), and a methylesterase (PME1), which both specifically act on an absolutely conserved C-terminal leucine (Leu309). Posttranslational modification of PP2A-C and its effect on holoenzyme assembly will be discussed in greater detail below.

**PP2A Regulatory B Subunits**

While PP2A-A and PP2A-C subunits generally are highly and ubiquitously expressed, regulatory B subunits generally are expressed at lower levels and in a more tissue-specific manner. This is in accordance with the role for PP2A-B subunits, which is to provide specificity for PP2A subcellular localization and substrate phosphoprotein targeting. Eukaryotic genomes encode up to four classes of regulatory subunits: B, B′, B″, and B‴ (reviewed in Janssens and Goris, 2001; Table 1). The Arabidopsis genome encodes at least 17 PP2A-B subunits, including members of the B, B', and B" families (Farkas et al., 2007). No Arabidopsis B''' family members have been identified to date (Table 1).

The *Saccharomyces cerevisiae* genome encodes two PP2A regulatory B subunits, *CDC55,* which belongs to the B family, and *RTS1,* a B' subunit (Table

1). Evidence supporting the model that B subunits direct PP2A substrate specificity comes from analysis of *cdc55Δ* and *rts1Δ* null mutants, which show distinct phenotypes. *cdc55Δ* strains exhibit cold sensitivity associated with microtubule and cell division defects, while *rts1Δ* null mutants are temperature sensitive. Interestingly, these mutant phenotypes cannot be rescued by overexpression of the remaining B subunit, indicating functional specialization (Shu et al., 1997; Zhao et al., 1997). Analysis of human PP2A-$B^B$ family members also supports the role for B subunits in directing subcellular localization of PP2A. In this case, Bα, Bβ, and Bε localize to the cytoplasm, while Bδ, Bγ1, and Bγ3 localize to the nucleus (McCright et al., 1996).

In addition to regulating the target specificity and subcellular localization of the holoenzyme, PP2A-B subunits are thought to be unstable in most organisms, requiring successful complex formation for protein accumulation. The exception to this is *Saccharomyces cerevisiae,* where the ratio of A:B:C is approximately 1:8:4. Rts1 protein is far more abundant than Cdc55 (~12:1). Therefore, the large molar excess of PP2A-B subunits is largely attributed to Rts1. In yeast, the large excess of B subunits suggests there may be alternate (non-PP2A) roles for these two proteins. Clearly, due to the high ratio of B: A/C, B subunits do not require successful PP2A holoenzyme formation for stability in yeast (Gentry and Hallberg, 2002; Janssens et al., 2008). In contrast, when regulatory B subunits are in molar excess to A and C, or when B subunits are restricted from holoenzyme entry, they are degraded in animals. Evidence for this comes from

*Drosophila* Schneider 2 cell experiments where ablation of either A or C subunits via RNAi resulted in the coordinate loss of B subunit accumulation (Silverstein et al., 2002). Additionally, prolonged knockdown of PPM1 expression in human cell lines prevents Leu309 methylation of PP2A-C (Longin et al., 2007). Because PP2A-$B^B$ subunits require PP2A-C methylation for binding, this abolishes the formation of PP2A-$B^B$ containing holoenzymes (Longin et al., 2007). PP2A holoenzymes containing PP2A-$B^B$ family members are necessary for cell viability in animals and plants (Longin et al., 2007; Janssens et al., 2008; Heidari et al., 2011). In human cells, prolonged PPM1 knockdown results in degradation of PP2A-$B^B$ subunits, suggesting that failure to enter a stable PP2A holoenzyme complex leads to PP2A-B degradation in mammalian cells (Longin et al., 2007).

**PP2A Catalytic Subunit Maturation and Holoenzyme Assembly**

Interestingly, in early studies of PP2A complexes purified from human erythrocytes and rabbit skeletal muscle, PP2A-C was not isolated alone but in distinct complexes, corresponding to an AC dimer, an ABC holoenzyme complex containing a 74 kDa B subunit, and an ABC holoenzyme complex containing a 53 kDa B subunit, which were all highly active against phosphosubstrates (Tung et al., 1985; Inoue et al., 1999). The physiological relevance of an active AC dimer, which is termed the “core dimer”, was questioned as a possible artifact of purification. However, using monoclonal antibodies that were raised against specific portions of the A subunit and were capable of recognizing free A, AC, and ABC enzyme complexes, it was shown that a population of active AC core

dimer does exist in a cellular context (Kremmer et al., 1997). This led to the question of how AC dimers remain free of regulatory B subunits *in vivo*, and how subsequent recruitment of regulatory B subunits is achieved.

An early report found that in contrast to AC dimers, holoenzyme complexes purified from bovine brain tissue were composed entirely of C-terminally methylated PP2A-C subunit, suggesting that after AC dimer formation, subsequent methylation by PPM1 is necessary to potentiate B subunit binding (Tolstykh et al., 2000). However, for the binding of human PP2A-$B^{B'gamma1}$, methylation of PP2A-C is not required *in vitro*, suggesting that there may be differential requirements for C-terminal methylation among PP2A-B subunits (Xu et al., 2006). Additionally, analysis of PP2A holoenzyme complexes formed in human cell lines has indicated that only the PP2A-$B^{B}$ family absolutely requires PP2A-C methylation for binding, while PP2A-$B^{B'}$ and PP2AB-$^{B''}$ family members can incorporate into either methylated or unmethylated AC dimers (Longin et al., 2007). It is now apparent that some, but not all B subunits require a Leu309 methylated PP2A-C for holoenzyme formation and that phosphorylation of PP2A-C Thr304 and Tyr307 can influence holoenzyme assembly as well (Janssens et al., 2008; Shi, 2009; see Figure 3).

Since PP2A normally functions not as a monomeric catalytic subunit, but as a holoenzyme containing two additional regulatory proteins (A + B) that constrain substrate specificity, the unregulated activity of nascently translated, unbound

catalytic subunit might pose a threat to cellular phosphoprotein homeostasis. In accordance with this, recent work has suggested that the catalytic subunit is folded in a low-activity conformation immediately following translation and prior to holoenzyme assembly (Fellner et al., 2003; Hombauer et al., 2007). In the low-activity state, the catalytic core of the enzyme is poorly structured to coordinate the two $Mn^{2+}$ ions required for enzyme function. It was recently shown that the chaperone-like activity of PTPA is required to fold PP2A-C into a fully active state. PTPA has an ATP-$Mg^{2+}$-dependent peptidyl-prolyl *cis/trans* isomerase activity that acts on Pro190 of PP2A-C to generate properly folded and active PP2A-C (Jordens et al., 2006; Leulliot et al., 2006).

The PP2A-C-activating function of PTPA is opposed by PME1. The structure of PME1 is interesting in that the monomeric protein is inactive as a methylesterase. Only upon complex formation with PP2A-C is the active site of PME1 rearranged to be fully functional (Xing et al., 2008). This is consistent with prior data indicating that although PME1 displays specificity towards PP2A-C, it is unable to demethylate a methylated peptide representing the PP2A-C C-terminus (Lee et al., 1996). Interestingly, it is not demethylation that inactivates PP2A-C subunits, but a structural rearrangement of the catalytic core that causes the loss of both coordinated $Mn^{2+}$ ions, resulting in a product that is similar to nascent, low-activity C (Xing et al., 2008). The structural studies of Xing and colleagues provide a high resolution explanation of previous reports that PME1-

inactivated PP2A-C was not reactivated by methylation using PPM1, but was reactivated upon incubation with PTPA (Longin et al., 2004).

PP2A-A subunits also play a prominent role in the generation of active PP2A-C. Yeast strains lacking the sole PP2A-A subunit, *tpd3Δ,* exhibit severely retarded growth which until recently was presumed to be a result of unrestricted PP2A-C activity. In fact, *tpd3Δ* strains have a two-fold reduction in bulk PP2A activity, which mirrors the Arabidopsis PP2A-A subunit mutant *rcn1* (Deruere et al., 1999; Hombauer et al., 2007). The loss of bulk PP2A activity in *tpd3Δ* and *rcn1* was relatively poorly understood until the prominent role that the A subunit plays in coupling PP2A holoenzyme assembly and PP2A-C maturation was proposed (see Figure 4). The full catalytic activation of PP2A-C requires the interaction of PTPA with the PP2A-A subunit (in the AC core dimer), which would explain why mutants lacking the structural subunit exhibit PP2A-defective phenotypes (Hombauer et al., 2007). The premature activation of PP2A-C is prevented by PME1, which inactivates and binds tightly to inactive C subunits until the holoenzyme assembly process proceeds (Ogris et al., 1997; Ogris et al., 1999; Hombauer et al., 2007).

Analysis of PP2A-A, PTPA, PPM1, and PME1 function in yeast has led to the formation of a model in which PP2A biogenesis is coupled to holoenzyme assembly (Hombauer et al., 2007, Figure 4). Linking PP2A biogenesis with holoenzyme assembly is an attractive notion since this could account for the

observed posttranslational regulation of PP2A at the activity level (Baharians and Schonthal, 1998; Lizotte et al., 1999). In this model, nascent PP2A-C is maintained in a low-activity conformation by the binding of PME1. PP2A-A also associates with the inactive PME1-PP2A-C complex. However, the molecular dynamics are still unclear. For example, does PME1 or PP2A-A bind to PP2A-C first? Further, what portion of PME1 activity is directed towards nascent PP2A-C versus preformed PP2A holoenzymes?

The next clearly defined step is the activation of PP2A-C by PTPA. PP2A-A also participates in this complex. Therefore the most parsimonious model for activating PP2A-C is that PME1 dissociates from the inactive PP2A core dimer and is replaced with PTPA, which catalyzes the activation of the core dimer (Figure 4). However, the molecular remodeling that takes place between the inactive complex containing PME1:PP2A-A:PP2A-C and the activating complex containing PTPA:PP2A-A:PP2A-C is unclear. Finally, after activation by PTPA, PPM1 methylates PP2A-C and the AC core dimer proceeds to form holoenzyme complexes with PP2A-B subunits (Figure 4).

Interestingly, PPM1 is required for full PP2A activity, with *ppm1Δ* strains showing similar defects in PP2A activity as either *ptpaΔ* or *tpd3Δ* (Hombauer et al., 2007). This is surprising given that only PP2A-$B^B$ regulatory subunits require PP2A-C methylation for entry into a holoenzyme. Therefore in yeast, only Cdc55 should require PP2A-C methylation. Interestingly, Rts1 is far more abundant than

Cdc55, suggesting that upon failure to methylate PP2A-C in *ppm1Δ* strains, the highly abundant Rts1 should still be able to incorporate into PP2A complexes. However, the *ppm1Δ* phenotype is far more severe than the *cdc55Δ* phenotype, suggesting that PPM1 functionality may include more than PP2A-C Leu309 methylation (Hombauer et al., 2007). This is in agreement with PP2A-C mutational analysis where deletion of Leu309 had a far weaker effect on PP2A activity than deletion of *ppm1Δ.*

It is thought that the presence or absence of C-terminal Leu309 methylation, in conjunction with two other C-terminal modifications, Thr304 and Tyr307 phosphorylation, can be read out as a sort of PP2A code, which will preferentially select for incorporation of B subunits compatible with the modifications present (reviewed in Janssens et al., 2008; see Figure 3). Posttranslational modifications to PP2A-C and their effects on holoenzyme formation have been best studied in yeast and mammalian model systems. However, the abundance and *in vivo* relevance of Thr304 and Tyr307 phosphorylation are still poorly understood in either system and very little is known about PP2A-C methylation and phosphorylation in plants. Interestingly, a recent report identified a mutant allele of Arabidopsis *PPM1* as a suppressor of *bri1* (*sbi1*; Wu et al., 2011). BRASSINOSTEROID INSENSITIVE1 (BRI1) is a receptor-like kinase that functions as the brassinosteroid receptor. Analysis of *sbi1* suggested that PP2A-C methylation controls PP2A subcellular localization and dephosphorylation of membrane-associated phosphoproteins (Wu et al., 2011).

## PP2A in *Arabidopsis thaliana*

PP2A has evolved roles in regulating plant-specific phenomena including the regulation of pivotal plant hormone biology such as ethylene biosynthesis, polar auxin transport, and responses to both abscisic acid and brassinosteroid (Garbers et al., 1996; Deruere et al., 1999; Larsen and Chang, 2001; Rashotte et al., 2001; Kwak et al., 2002; Larsen and Cancel, 2003; Muday et al., 2006; Pernas et al., 2007; Tang et al., 2011; Wu et al., 2011; Figure 5). The fact that PP2A regulates the production, transport, and/or perception of so many growth regulators indicates the central role that PP2A has evolved in regulating plant-specific development. Much of our current understanding of PP2A in Arabidopsis has come from the study of one PP2A mutant, *rcn1*, which exhibits a two-fold drop in PP2A activity (Deruere et al., 1999). The *rcn1* mutant exhibits pleiotropic defects resulting from the misregulation of developmental and metabolic pathways, but retains viability and fertility, making it a valuable tool for the study of processes dependent on PP2A activity. Treatment of wild-type plants with PP2A inhibitors produces a phenocopy of *rcn1,* confirming that the *rcn1* mutation results in a partial PP2A loss-of-function (Deruere et al., 1999).

## PP2A Subunits Encoded in the Arabidopsis Genome

The Arabidopsis genome encodes three scaffolding A subunits, five catalytic C subunits, and at least 17 regulatory B subunits (Table 1). This diversity of subunits can give rise to as many as 255 unique PP2A holoenzymes, illustrating

the composite nature of total cellular PP2A activity (DeLong, 2006). The three scaffolding subunits in Arabidopsis are *RCN1* (At1g25490), *PP2AA2* (At3g25800), and *PP2AA3* (At1g13320). Despite ubiquitous expression patterns and high sequence similarity between the 3 Arabidopsis A subunit isoforms, *rcn1* mutant plants have reduced PP2A activity and pleiotropic defects, but neither single mutants in either of the other two Arabidopsis PP2A-A subunit genes (*pp2aa2* and *pp2aa3*) nor the double mutant *pp2aa2 pp2aa3* exhibit these obvious defects (Zhou et al., 2004). Considering the importance of the scaffolding A subunit in PP2A holoenzyme assembly and maturation of PP2A-C, this might reflect a specific role for the RCN1 scaffold in the proper maturation of PP2A complexes in Arabidopsis.

In addition to uncovering the role RCN1 plays in promoting PP2A activity, single and double mutant analysis of Arabidopsis PP2A-A subunits has suggested that each subunit, although constitutively and ubiquitously expressed (Czechowski et al., 2005), preferentially regulates a specific subset of physiological processes (Zhou et al., 2004; Blakeslee et al., 2008). An example of PP2A-A subunit specialization is the observation that double mutant *rcn1 pp2aa2* and *rcn1 pp2aa3* plants are both severely dwarfed, but only the *rcn1 pp2aa2* plants are female sterile (Zhou et al., 2004). This may be due to differential affinity for incorporation of specific PP2A-B subunits into different RCN1-C, PP2AA2-C, and PP2AA3-C core dimers. Many animal genomes encode two isoforms of the A subunit, $\alpha$ and $\beta$. In humans, A$\alpha$ is expressed at far higher levels than A$\beta$.

However, each PP2A-A isoform has been shown to interact with unique regulatory B subunits and specific lesions in each A subunit isoform have been associated with tumor development due to the misregulation of distinct dephosphorylation events (Moreno et al., 2004; Sablina et al., 2007). The expanded repertoire of three A subunit isoforms in Arabidopsis and the unique roles that each has evolved, especially the possible role RCN1 plays in activating PP2A-C, provide a good model system to study PP2A-A subunit redundancy and specialization.

**Isolation of the *rcn1-1* Mutant**

The *rcn1-1* mutant was originally identified in a forward genetic screen for Arabidopsis seedlings with an altered root growth phenotype on media containing the auxin transport inhibitor 1-Naphthylphthalamic Acid (NPA; Garbers et al., 1996). Indole-3-acetic acid (IAA), the most biologically significant auxin *in planta* is primarily biosynthesized at the shoot apical meristem and transported downward to the root tip. At the root tip, the auxin transport stream is redirected away from the root tip, a process known as basipetal auxin transport (Figure 5A). The directional transport of auxin creates an auxin gradient, with a local maximum near the root tip that is important for several aspects of root biology, including maintenance of a stem cell population and differential growth responses upon gravity stimulation (reviewed in Blakeslee et al., 2005; Bennett and Scheres, 2010). Since auxin regulates asymmetric cell elongation, application of the auxin transport inhibitor NPA reduces growth curvatures such

as gravitropism. The *rcn1* mutant was identified based on a characteristic root curling phenotype, leading to the name, *roots curl in 1-naphthylphthalamic acid 1* (*rcn1* Garbers et al., 1996). The loss of NPA-mediated inhibition of root gravitropism in *rcn1* plants is at least partially due to increased basipetal auxin transport compared to wild-type seedlings (Rashotte et al., 2001). More recent analyses of auxin transport have shown that PP2A and the PINOID (PID) kinase antagonistically regulate the phosphorylation status of PINFORMED (PIN) auxin transport proteins (Michniewicz et al., 2007; Sukumar et al., 2009). PIN proteins transport auxin in a directional manner dependent on differential localization to the plasma membrane (Blakeslee et al., 2005). The phosphorylation of PINs is thought to regulate vesicular transport and deposition and recycling of PIN proteins from the membrane, thereby modulating auxin transport.

**A Role for RCN1 in Ethylene Production and Perception**

A second mutant allele of *RCN1* was identified in a screen for *enhanced ethylene response (eer)* Arabidopsis mutants. The *eer1* mutant exhibits short and thick hypocotyls in the absence of ethylene and an exaggerated hypocotyl response upon application of exogenous ethylene or ACC (Larsen and Chang, 2001). Mapping of the *eer1* locus revealed a loss-of-function mutation in *RCN1* and *eer1* was designated *rcn1-2* (Larsen and Cancel, 2003). Both *rcn1* and *eer1* are complete loss-of-function alleles of *RCN1* and display the same phenotypes.

The short hypocotyl phenotype of dark-grown *rcn1* plants is largely the result of ethylene overproduction (Larsen and Chang, 2001; Muday et al., 2006). The *rcn1* hypocotyl elongation defect is suppressed by *etr1* and *ein2,* mutations that impede ethylene perception. Additionally, treatment with AVG to block ethylene production restores hypocotyl elongation (Figure 5B). Interestingly, *rcn1* and wild-type seedlings produce comparable levels of ethylene when grown in the light, indicating that the ethylene overproduction phenotype of *rcn1* seedlings is specifically linked to growth in the dark (Muday et al., 2006). In fact, wild-type seedlings produce significantly less ethylene when grown in the dark, compared to growth in the light. This down-regulation of ethylene biosynthesis during dark-growth allows for proper hypocotyl elongation and emergence from the soil. The levels of ethylene production in *rcn1* seedlings grown in either light or dark conditions are the same as wild-type ethylene production in the light (Muday et al., 2006). This indicates that *rcn1* seedlings fail to limit ethylene production when grown in the dark, leading to an overproduction of ethylene when compared to wild-type plants (Muday et al., 2006).

Some aspects of the *rcn1* phenotype suggest effects on ethylene signaling. For example, when *rcn1* seedlings are grown in the presence of AVG (which inhibits endogenous ethylene production) and treated with exogenous ethylene they exhibit increased ethylene responsiveness compared to wild-type plants (Larsen and Chang, 2001). Furthermore, *rcn1* shows a complex genetic interaction with at least one ethylene response component. CONSTITUTIVE ETHYLENE

RESPONSE1 (CTR1) is a putative MAPKKK that functions to repress ethylene signaling in the absence of ethylene. Etiolated loss-of-function *ctr1* seedlings exhibit an extremely short hypocotyl, short root, and exaggerated apical hook associated with constitutive ethylene response. When crossed with *rcn1,* the *ctr1 rcn1* double mutant exhibits an enhanced hypocotyl elongation defect, suggesting additivity, but surprisingly, a longer root than the *ctr1* single mutant indicating suppression of the *ctr1* signaling defect (Larsen and Cancel, 2003). This suggests that PP2A inputs into ethylene signaling may be tissue specific. Thus, the effect of the *rcn1* mutation on ethylene perception is complicated and the mechanism underlying regulation is unclear.

**The Gaseous Plant Growth Regulator Ethylene**

The production and physiological effects of the gaseous hormone ethylene have been subjects of intense study since ethylene was first identified as the causative agent of defoliation in illuminating gas a century ago (Neljubov, 1901; reviewed in Abeles, 1992). Ethylene regulates diverse physiological processes throughout plant development, including seed germination, root hair formation, abiotic and biotic stress responses, fruit ripening, and organ senescence and abscission (reviewed in Yang, 1984; Abeles, 1992; Schaller, 2002). Ethylene is also a potent inhibitor of cell expansion in nearly all tissues and growth conditions (reviewed in Johnson and Ecker, 1998). Two notable exceptions to this are light-grown Arabidopsis hypocotyls, where ethylene stimulates hypocotyl elongation, and deepwater rice, where ethylene signaling promotes shoot elongation upon

flooding (Kende, 1998; De Paepe and Van der Straeten, 2005; Hattori et al., 2009).

Since ethylene inhibits hypocotyl elongation in dark-grown seedlings, several genetic screens have been designed to identify mutants with altered biosynthesis or perception of ethylene based on hypocotyl phenotypes. These screens have identified many of the components involved in ethylene perception and signaling (Guzman and Ecker, 1990; reviewed in Bleecker and Kende, 2000). This approach also identified three *ethylene overproducer (eto)* mutants that all increase the protein stability of a specific class of enzymes in the ethylene biosynthetic pathway. Identification of the *ETO* mutants underscores the importance of the ethylene biosynthetic pathway and the prominent role played by regulated protein stability in controlling ethylene levels (see below).

**The Ethylene Biosynthetic Pathway**

Ethylene is biosynthesized from the common precursor, S-adenosyl-methionine (SAM) in two committed enzymatic reactions (Figure 6). First, SAM is converted to 1-aminocyclopropane-1-carboxylate (ACC) by the enzyme ACC synthase (ACS). A byproduct of ACC production is 5'-methylthioadenosine, which is recycled into methionine by the Yang Cycle (Miyazaki and Yang, 1987). The final step in the pathway is catalyzed by ACC oxidase (ACO), which oxidatively cleaves ACC to form ethylene, cyanide, and $CO_2$. Cyanide is detoxified by β–cyanoalanine synthase (Yip and Yang, 1988). The salvage of methionine and

detoxification of cyanide allow for high levels of ethylene production without depletion of methionine stores or buildup of toxic byproducts (Figure 6).

In higher plants, multi-gene families encode both ACS and ACO, reflecting the importance of maintaining both specialized and tight control over ethylene production. The Arabidopsis genome encodes nine ACS enzymes, eight of which are active (ACS2, 4, 5, 6, 7, 8, 9, and 11) and one enzymatically inactive isoform (ACS1; Yamagami et al., 2003). In contrast to some plant hormones, ethylene can be produced in nearly all tissues. In most tissues and developmental windows ACS activity is rate-limiting for ethylene production. For this reason, the expression levels, enzymatic activities, and posttranslational regulation of ACS enzymes have been subjects of extensive analysis (reviewed in Chae and Kieber, 2005; De Paepe and Van der Straeten, 2005; Argueso et al., 2007; Lin et al., 2009).

ACS enzymes are pyridoxal phosphate-dependent and highly related to subgroup 1 aminotransferases (Rottmann et al., 1991). Similarly to aminotransferases, ACS enzymes function as dimers (Tarun and Theologis, 1998). In Arabidopsis, and other species, including tomato (*Solanum lycopersicum,* formerly *Lycopersicum esculentum*), ACS isozymes group into two clear phylogenetic clades (Tsuchisaka and Theologis, 2004; Argueso et al., 2007). In Arabidopsis, ACS1, 2, and 6 form one phylogenetic group, and ACS 4, 5, 7, 8, 9, and 11 form the other (Figure 7). Phylogenetic clustering correlates

well with biochemical data indicating that ACS enzymes form functional heterodimers only with other members from the same clade. The one exception to this is ACS7, which forms catalytically active heterodimers with members of both clades (Tsuchisaka and Theologis, 2004). Interestingly, while inactive as a homodimer, ACS1 can form functional heterodimers with either ACS2 or 6, providing an additional layer of ACS regulation (Yamagami et al., 2003; Tsuchisaka and Theologis, 2004; Tsuchisaka et al., 2009).

ACS enzymes have been further classified into three types, based on the presence or absence or C-terminal regulatory motifs (Figure 8, Table 3). However, the placement of ACS11 has been controversial, which may indicate the oversimplification of grouping ACS enzymes into only three types (Yoshida et al., 2005; Argueso et al., 2007). The current paradigm for placement of Arabidopsis ACS family members into each type is as follows. Type 1 ACS enzymes (ACS1, 2, and 6) exhibit the longest C-termini, consisting of two regulatory motifs, a mitogen-activated protein kinase (MAPK) phosphorylation target motif containing three conserved serine target residues (Liu and Zhang, 2004), as well as a sequence variant of a calcium-dependent protein kinase (CDPK) consensus phosphorylation target motif that contains a single serine phosphorylation site (Hernandez Sebastia et al., 2004). Type 2 enzymes ACS 4, 5, 8, and 9 have shorter C-termini, containing only the putative CDPK phosphorylation motif. The type 3 enzymes ACS7 and ACS11 possess the shortest C-termini and until recently, these isozymes were not expected to be

phosphoregulated (Yoshida et al., 2006; Figure 8). However, the ACS11 C-terminus is longer than ACS7 and shows some sequence similarity to type 2 ACSs (Figure 7).

To date, CDPK-mediated phosphoregulation of ACS isozymes has been substantiated only in tomato (Tatsuki and Mori, 2001; Kamiyoshihara et al., 2010). Recent work in tomato has shown that the type 1 ACS isozyme Le-ACS2 requires full phosphorylation of both MAPK and CDPK target sites to stabilize the protein against proteasome-dependent turnover (Kamiyoshihara et al., 2010). In contrast, type 1 enzymes in Arabidopsis are stabilized by the triple phosphorylation of the MAPK target site alone and phosphoregulation of the putative CDPK site is uncertain (Liu and Zhang, 2004; Joo et al., 2008). The CDPK target site in Arabidopsis type 2 enzymes is a non-canonical motif, but can be phosphorylated *in vitro* by CDPK protein kinase fractions extracted from *Zea maize* (Hernandez Sebastia et al., 2004).

**Posttranslational Regulation of ACS Enzyme Activity**

In addition to tight transcriptional regulation, a major determinant of total ethylene biosynthesis is the posttranslational modification of ACS enzyme stability. Type 1 and 2 ACS enzymes are normally short-lived proteins, with half-lives that can be increased in response to various developmental, hormonal, and environmental cues to stimulate ethylene biosynthesis. The rapid turnover of ACS enzymes was initially investigated in tomato, where the half-life was

determined to be 20 minutes in leaves and 40 minutes and 2 hours in green and pink tomato fruit, respectively (Kende, 1981; Spanu et al., 1990). This work helped establish the paradigm in which the stability of ACS enzymes influences overall levels of ethylene production.

Work with tomato suspension cultures showed that ACS activity could be rapidly increased upon treatment with a fungal elicitor, mimicking *in planta* biotic stress stimulation of ethylene synthesis (Felix et al., 1994). The elicitor-induced increase in ACS activity was sensitive to regulation by protein phosphorylation and dephosphorylation, since treatment with K-252a (a general protein kinase inhibitor) blocked the elicitor-induced ACS activity increase, while treatment with calyculin A (a protein phosphatase inhibitor) increased ACS activity without elicitor treatment (Felix et al., 1994; Spanu et al., 1994). Elicitor- and phosphorylation-dependent increases in ACS activity were also sensitive to treatment with cycloheximide, indicating that protein turnover (a short half-life) plays a large part in modulating ACS activity (Spanu et al., 1994). Recent genetic and biochemical studies have refined our knowledge of the kinases and phosphatases involved in regulating elicitor-stimulated ethylene biosynthesis, which results from phosphorylation-induced stabilization of type 1 ACS enzyme activity.

**Posttranslational Regulation of Type 1 ACS Isozymes**

The phosphostabilization of type 1 ACS enzymes has been studied in both tomato and Arabidopsis. In tomato, a wound-inducible ACS isozyme, LeACS2, is phosphorylated at Ser460 upon wounding stimulation in fruit (Tatsuki and Mori, 2001). This site is orthologous to the CDPK phosphorylation motif found upstream of MAPK phosphorylation sites of Arabidopsis type 1 ACS isozymes (Figure 9). LeACS2 was the first ACS enzyme to be confirmed as a phosphoprotein *in vivo*. In tomato fruit, phosphorylation of both the CDPK target and downstream MAPK target motifs is required for the stability of LeACS2 (Kamiyoshihara et al., 2010). In that study, the authors were unable to detect appreciable quantities of either singly phosphorylated or unphosphorylated LeACS2, indicating that these species are rapidly degraded. This suggests that nascent LeACS2 must be rapidly phosphorylated by both CDPKs and MAPKs to avoid turnover (Kamiyoshihara et al., 2010). Several studies have implicated protein phosphatases as important negative regulators of stress-induced increases in ACS activity (Felix et al., 1994; Spanu et al., 1994; Kamiyoshihara et al., 2010). The fact that treatment with protein phosphatase inhibitors (e.g. okadaic acid and calyculin A) increases ACS activity shows that there is constant action by phosphatases to maintain low basal ACS activity in the absence of elicitor stimulation. However, the relatively high doses of phosphatase inhibitors that have been used in these studies would inhibit a broad spectrum of plant phosphatases, including PP1, PP2A, PP4, PP5, and PP6 (Farkas et al., 2007;

see Table 1). Until recently, this has precluded the identification of a specific phosphatase that regulates ACS activity.

Work from Shuqun Zhang's lab demonstrated that activation of an evolutionarily conserved stress-induced MAPK cascade culminates with the phosphorylation of type 1 ACS isozymes (Kim et al., 2003; Liu and Zhang, 2004). This work provides clear causal linkage between stress activation of MAPKs and ethylene biosynthesis. In Arabidopsis, activation of MKK4/5 by an-as-yet unidentified upstream MAPKKK leads to the downstream activation of MPK3/6, which in turn phosphorylate ACS2/6 (and presumably ACS1 as well; Figure 8). In fact, the C-terminal MAPK phosphorylation motif of ACS6 was the first confirmed *in vivo* target of MAPK phosphorylation in plants (Liu and Zhang, 2004). In contrast to LeACS2, which apparently requires phosphorylation of all MAPK and CDPK sites for maximal stability, in Arabidopsis, phosphorylation of the three clustered MAPK target sites alone is sufficient to stabilize ACS6 (Liu and Zhang, 2004; Joo et al., 2008). The 16 C-terminal residues of ACS6 constitute the degron that targets type 1 ACS enzymes to the proteasome as these residues are required confer instability (Joo et al., 2008). Curiously, this degron is downstream of the putative CDPK phosphorylation motif, further suggesting that CDPK phosphorylation is not a major factor regulating the stability of Arabidopsis type 1 ACS isozymes.

The only protein phosphatase conclusively established in regulating ethylene biosynthesis is the PP2C, AP2C1 (Schweighofer et al., 2007). AP2C1 contains a kinase interaction motif (KIM), which is common among MAPK phosphatases. In agreement with this, AP2C1 interacts with both MPK4 and MPK6 and inactivates them through dephosphorylation (Schweighofer et al., 2007). MPK6 plays a well-characterized role in promoting stress-induced ethylene biosynthesis (Liu and Zhang, 2004; Joo et al., 2008; Han et al., 2010). Transgenic plants overexpressing AP2C1 produce less ethylene in response to wounding than wild-type plants (Schweighofer et al., 2007). This indicates that AP2C1 negatively regulates ethylene biosynthesis indirectly through inactivating MPK6.

Several questions remain concerning the phospho-regulated stability of type 1 ACS enzymes. For example, what is the role of the putative CDPK motif in Arabidopsis? What is the role of RCN1/PP2A in regulating ethylene biosynthesis? Furthermore, the mechanism through which non-phosphorylated type 1 ACS are recognized and shuttled to the proteasome for degradation is unknown. It is likely that ubiquitination serves as an important control step in turnover of type 1 ACS. However, to date, high molecular weight polyubiquitinated type 1 ACS isozymes have not been detected. Also, the putative E3 ubiquitin ligase(s) responsible for type 1 ACS recognition and turnover has yet to be identified, through either genetic or biochemical experiments.

**Posttranslational Regulation of Type 2 ACS Isozymes**

Three *ethylene overproducer (eto)* mutants have been isolated in Arabidopsis and their molecular characterization has increased our understanding of the posttranslational regulation of type 2 ACS stability. The recessive *eto1* mutant was originally isolated in a screen for constitutively responding mutants and was shown to produce 40-fold more ethylene than wild-type plants (Guzman and Ecker, 1990). Subsequent characterization of ETO1 demonstrated that it is a Broad-complex, Tram track, Bric-a-brac (BTB) containing E3-ubiquitin ligase substrate specificity adaptor that interacts with Cul3A and B (Wang et al., 2004). Two closely related BTB proteins, ETO1-LIKE1 (EOL1) and ETO1-LIKE2 (EOL2) are partially redundant with ETO1 (Yoshida et al., 2006). ETO1 specifically recognizes a C-terminal sequence (in type 2 ACS isozymes) termed the Target Of Eto1 (TOE) degron which targets ACS isozymes for ubiquitination and subsequent degradation by the 26S proteasome (Yoshida et al., 2005; Yoshida et al., 2006). In this manner, ETO1, EOL1, and EOL2 all recognize type 2 ACS enzymes (but not type 1 or type 3) enzymes and regulate their turnover (Yoshida et al., 2006; Christians et al., 2009; see Table 3). An unrelated E3 also acts on ACS proteins; the RING domain-containing E3 ubiquitin ligase XBAT32 interacts with ACS4 (a type 2 ACS isozyme) and ACS7 (a type 3 ACS isozyme) and is able to ubiquitinate both ACS4 and ACS7 *in vitro* (Prasad et al., 2010). Interestingly, XBAT32 does not interact with type 1 ACS isozymes, reminiscent of ETO1 function (Prasad et al., 2010; Table 3).

Two dominant *eto* mutants were isolated, *eto2* and *eto3,* that carry lesions in the C-termini of two type 2 ACS proteins, ACS5 and ACS9, respectively (Kieber et al., 1993). Both of these *eto* mutations alter the TOE sequence and allow for increased protein stability and ACS activity due to the loss of ETO1 surveillance (Chae et al., 2003; Christians et al., 2009). Intriguingly, the TOE sequence of type 2 enzymes overlaps with the putative CDPK target site. $ACS5^{eto2}$ is the result of a frameshift mutation that eliminates both the TOE sequence and the CDPK motif (Vogel et al., 1998). The $ACS9^{eto3}$ mutation also disrupts the TOE, but results from a single amino acid substitution, Val 257 to Asp (Chae et al., 2003; Yoshida et al., 2006). This substitution occurs four amino acids N-terminal to the putative CDPK target serine residue, and is not predicted to affect the CDPK target motif. However, it might function as a CDPK site phosphomimic.

The relevance of CDPK phosphorylation to the regulation of type 2 ACS protein stability remains uncertain. Interestingly, substituting either Ala or Asp for the putative CDPK target Ser461 of ACS5 did not affect the ability of ACS5 to interact with ETO1 or EOL1 in a yeast two-hybrid assay (Christians et al., 2009). However, the *in planta* significance of these substitutions has yet to be determined. To date, all of the data suggesting that Arabidopsis type 2 ACS enzymes might be targets of CDPK action comes from *in vitro* analysis (Hernandez Sebastia et al., 2004), and the direct effect of phosphorylation on the stability of type 2 enzymes has yet to be determined *in planta*. Although LeACS2 is a type 1 enzyme, the finding that (in addition to MAPK phosphorylation) CDPK

phosphorylation is crucial for stress-induced LeACS2 stability does provide a precedent for *in planta* CDPK regulation of ACS proteins (Kamiyoshihara et al., 2010).

**Posttranslational Regulation of Type 3 ACS Isozymes**

Until recently, there was no evidence for, or suggestion of posttranslational regulation of the type 3 ACS enzymes. However, ACS6, 7, and 8 were identified in a screen for 14-3-3 omega interacting proteins (Chang et al., 2009). Normally, 14-3-3 proteins function as dimers, binding to client proteins in a phosphorylation-dependent manner. Thus, identification of a representative member from each ACS isozyme type indicates that all ACS isozymes may be regulated through phosphorylation. Additionally, the identification of ACS7 as a putative 14-3-3 client suggests that phosphoregulation might occur in the highly conserved catalytic domain ACS. This putative phosphorylation event could be important for localization, activity, turnover, or even regulation of ACS dimerization.

Ethylene biosynthesis is controlled at multiple levels, introducing many possible points where PP2A could exert regulatory action. The ACS family of enzymes is highly regulated. In Arabidopsis, phosphorylation of ACS isozymes by MAPKs and CDPKs play known and putative roles in modulating ethylene biosynthesis, respectively. Additionally, phosphorylation within the conserved N-terminus of ACS isozymes could regulate enzyme function. The ETO1 and EOL proteins

regulate ethylene biosynthesis by controlling type 2 ACS isozyme turnover and are putative phosphoproteins *in vivo.* The MAPK cascade that stabilizes type 1 ACS isozymes could also be regulated by dephosphorylation of signaling components. Importantly, the determinants responsible for directing PP2A action on ethylene biosynthesis are poorly understood.

**Analysis of Arabidopsis PP2A Functions and Regulation *in vivo***

The disparate phenotypes for each of the three PP2A-A subunit mutants, where *rcn1* exhibits pleiotropic defects while *pp2aa2* and *pp2aa3* mutants appear phenotypically wild-type, and the unique regulatory roles that are unmasked when either *pp2aa2* or *pp2aa3* are in combination with *rcn1* is striking (Zhou et al., 2004). This is especially interesting because the A2 and A3 proteins share 94% sequence identity with each other and 86% sequence identity with the RCN1 protein and because all three subunits, at a gross level, are highly and ubiquitously expressed (Zhou et al., 2004). This indicates that either a relatively small number of amino acids are responsible for significant functional differences, possibly through differential interaction with PP2A-C maturation factors or regulatory B subunits. Alternatively, variations in tissue-specific accumulation patterns could account for the phenotypic differences observed.

In order to dissect the relative contributions of expression patterns and protein sequence for RCN1 and PP2AA3, a combination of promoter-coding sequence fusion constructs were generated and transformed into *rcn1* mutant plants.

Rescue of *rcn1* mutant phenotypes was measured for transgenic plants harboring the constructs; $RCN1_{pro}$:YFP-RCN1, $RCN1_{pro}$:RCN1:YFP, $RCN1_{pro}$:YFP-PP2A-A3, and PP2A-$A3_{pro}$:YFP-PP2A-A3. Surprisingly, we found that any increase in A subunit dosage in *rcn1* plants resulted in rescue of the dark-grown short-hypocotyl phenotype. This finding indicates that RCN1-specific amino acid sequences are not required for proper regulation of ethylene biosynthesis in the dark. In contrast, the rescue of root disorganization specifically required RCN1 coding sequences, indicating a specialized role for RCN1-mediated dephosphorylation in the maintenance of the root tip auxin maximum and cellular division and development. This work is presented in Chapter 2, Specificity of RCN1-Mediated Protein Phosphatase 2A Regulation in Meristem Organization and Stress Response in Roots (Blakeslee et al., 2008).

Ethylene production is largely controlled through modulating the protein stability of the rate-limiting enzyme in ethylene biosynthesis, ACC synthase (ACS). PP2A was implicated in regulation of ethylene synthesis when the PP2A-deficient *rcn1* mutant was shown to overproduce ethylene. My thesis work was designed to test the hypothesis that sustained hyperphosphorylation of one or several classes of ACS isozyme(s) due to decreased PP2A activity might account for ethylene overproduction in *rcn1* plants. Using a combination of genetic and biochemical techniques, I showed that *rcn1* plants overproduce ethylene because one specific class of ACS enzymes, the type 1 ACS, are hyperphosphorylated and have increased stability. Additionally, PP2A interacts

with ACS6, a type 1 enzyme, and PP2A complexes dephosphorylate an ACS6 phosphopeptide *in vitro* suggesting that type 1 ACS enzymes are bona fide PP2A substrates *in planta*. Interestingly, I discovered an unexpected role for PP2A in promoting the stability of type 2 ACS enzymes. PP2A interacts with ACS5, a type 2 isozyme in co-immunoprecipitation experiments, suggesting that PP2A may directly regulate type 2 isozymes. Our analysis of PP2A-mediated ethylene production illustrates the finely tuned and phosphorylation-dependent regulation of ethylene synthesis. The main body of work for my Ph.D. thesis revolves around the PP2A regulation of ethylene biosynthesis and is presented in Chapter 3, Protein Phosphatase 2A Controls Ethylene Biosynthesis by Differentially Regulating the Turnover of ACC Synthase Isoforms (Skottke et al., 2011) and Appendix 2, The Quest for Additional PP2A Substrates.

It is now evident that several proteins need to interact with nascent PP2A-C in order to assemble a pool of functional holoenzymes. Mainly through work in yeast, we know that PME1 interacts stably with a pool of inactive C subunit and that this interaction serves as a PP2A quality control mechanism. PME1 stably inactivates PP2A-C, using a metal ion displacement mechanism to disrupt the catalytic center until the binding and action of PP2A-A and PTPA allows for proper C subunit refolding, metal ion coordination, and full catalytic activity (see Figure 4). Although many aspects of PP2A biology are absolutely conserved throughout eukaryotes, there are key differences between yeast and other eukaryotes. A prime example of this is the differential regulation of B subunit

accumulation, where yeast have a stoichiometric excess of B compared to A and C, whereas excess B that is not incorporated into functional holoenzymes in animals is degraded. Although PME1, PPM1, and PTPA are all present and highly conserved in eukaryotes, the question of whether eukaryotes other than yeast use a C subunit maturation cycle that is coupled to holoenzyme assembly remains unanswered. Characterization of an Arabidopsis *pme1-1* mutant has revealed that plants most likely regulate PP2A holoenzyme biogenesis and assembly in a manner similar to yeast and that this mechanism is conserved in eukaryotes. The fact that *rcn1* but not *pp2aa2, pp2aa3,* or *pp2aa2 pp2aa3* seedlings show a defect in total cellular PP2A activity suggests that RCN1 may specifically participate in PP2A-C activation / holoenzyme biogenesis. Further characterization of transgenic plants harboring RCN1 and PP2AA3 promoter-coding sequence fusion constructs demonstrates that PP2A-A subunit dosage regulates PP2A activity within a defined window. The analysis of PP2A-A dosage contributions to physiologically relevant PP2A activity is presented in Appendix 1, Regulation of Arabidopsis PP2A activity *in vivo.*

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**FIGURES**

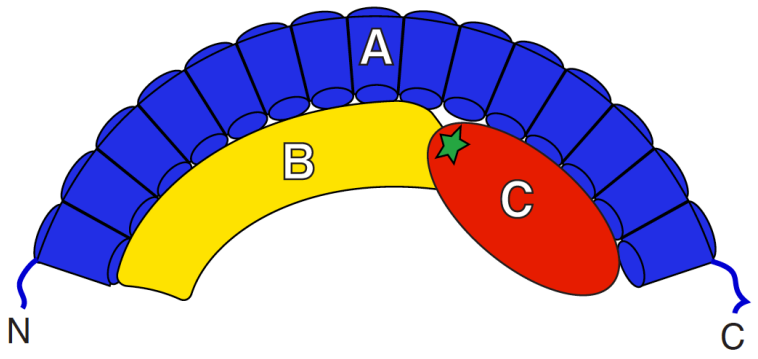



**Figure 1. Schematic representation of the protein phosphatase 2A holoenzyme complex.** PP2A functions *in vivo* as a heterotrimeric holoenzyme complex. Complex assembly is mediated by the scaffolding A subunit, shown in blue, which provides a hydrophobic interaction surface for the recruitment of a regulatory B subunit, shown in yellow, and the catalytic C subunit, shown in red. Holoenzyme assembly juxtaposes the regulatory B subunit, which controls PP2A substrate specificity and subcellular localization, and the active site (green star). This is thought to ideally position phosphosubstrates for dephosphorylation.

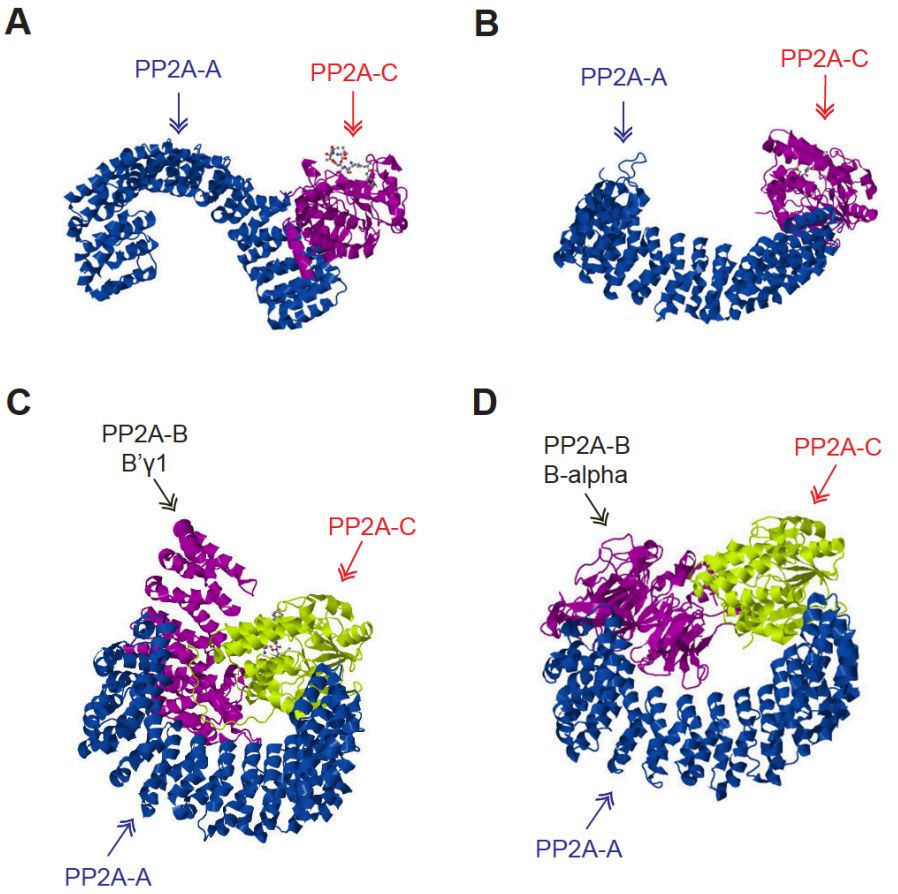



**Figure 2. Structures of the PP2A-AC core dimer and two holoenzyme complexes.** (A, B) Two views of the PP2A core dimer, consisting of the human Aα and Cα subunits (Xing et al., 2006). In panel A, the horseshoe shape of the PP2A-A subunit is apparent, as well as the interaction between PP2A-C and the intraloop ridge of PP2A-A. The small molecule inhibitor microcystin is shown in a ball and stick representation. The AC core dimer view shown in panel B more closely resembles the orientation of the holoenzyme complexes presented in panels C and D. (C) Structure of the PP2A holoenzyme complex composed of human Aα, Cα, and B'γ1 (Xu et al., 2006). Like PP2A-A, B'γ1 is composed of a series of 8 tandem HEAT repeats. In this structure B'γ1 makes substantial intermolecular contacts with both the A and C subunits. (D) Structure of the PP2A holoenzyme complex composed of human Aα, Cα, and Bα (Xu et al., 2008). The structure of Bα primarily contains β-sheets and differs substantially from the structure of B'γ1, which is in accordance with the differential regulatory roles of B subunits. Blue arrows indicate the PP2A-A subunit, red arrows the PP2A-C subunit, and black arrows the PP2A-B subunit. All structural information was retrieved from the protein data bank and visualized using Jmol.

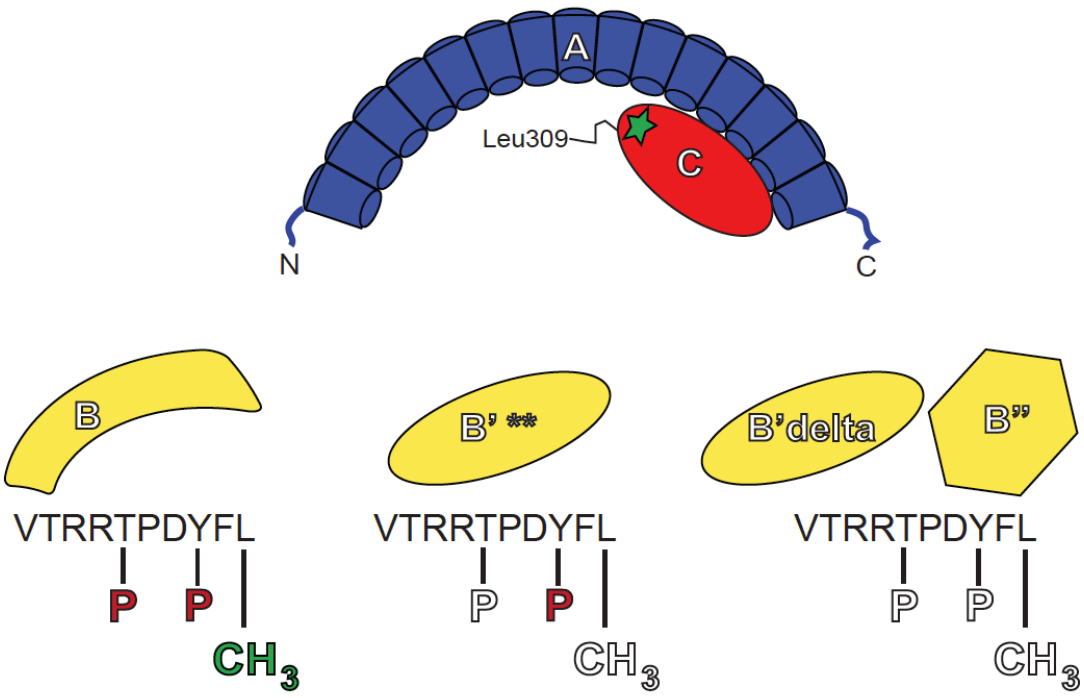



**Figure 3. The PP2A-C code.** This schematic represents how modification of the C-terminal tail of PP2A-C can lead to differential association with regulatory B subunits. The VTRRTPDYFL sequence corresponds to the 10 C-terminal amino acids of PP2A-C. Methylation of Leu309 is indicated by "Me", whereas phosphorylation of Thr304 and Tyr307 is shown as a "P". Phosphorylation and methyl marks are red when the indicated modification is inhibitory for complex formation. The PP2A-B B family is the only class of B subunits that require Leu309 methylation for holoenzyme assembly (Longin et al. 2009). In addition, phosphorylation of both Thr304 is inhibitory. In contrast, the PP2A-B B' and PP2A-B B" families exhibit no known preference for C-terminal PP2A-C modification. The role of PP2A-C C-terminal methylation and phosphorylation have been most extensively studied in animals and little information is available concerning the regulatory importance of these modifications in plants. B'** refers to all B' members except B'delta.

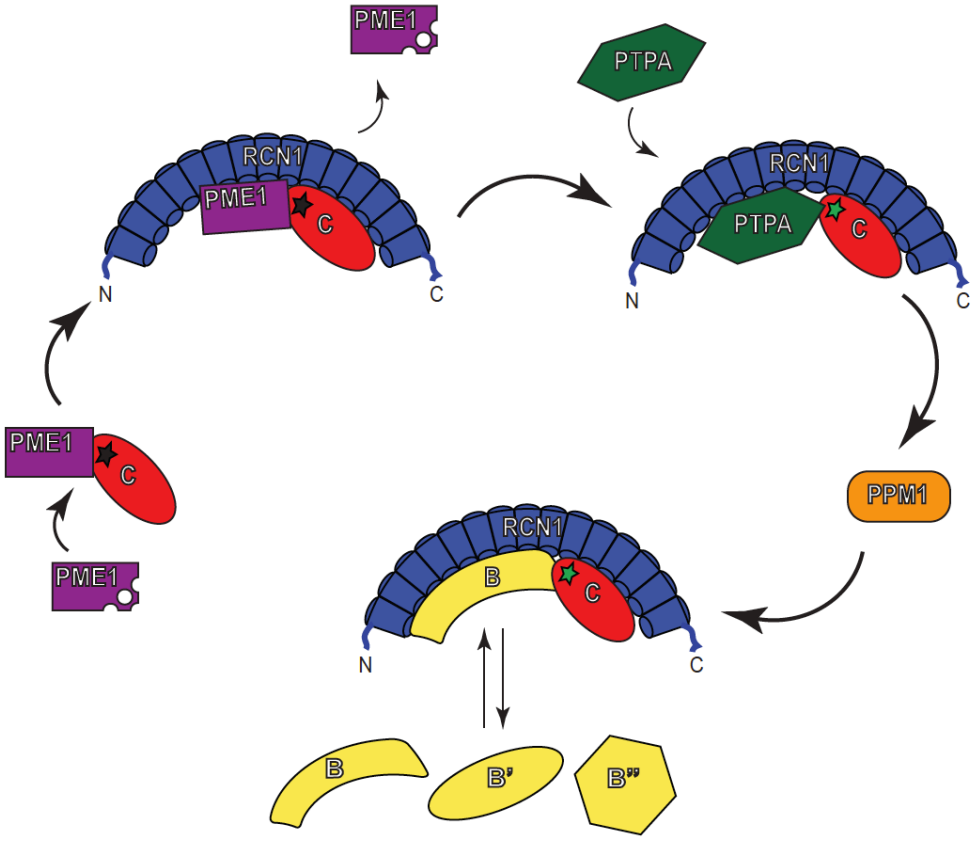



**Figure 4. Model of how C subunit activation is coupled to PP2A holoenzyme assembly.** In this model, nascent, low activity PP2A-C is represented with a black star in the catalytic site. Low activity C is bound and kept inactive by PME1. PME1 is non-functional as an apo enzyme (represented as "swiss cheese" PME1), but gains full activity upon binding to the PP2A-C subunit. PME1, low-activity PP2A-C, and PP2A-A all associate in a complex (Ogris et al., 1999). Presumably upon cellular demand for increased PP2A activity, PME1 releases from the complex and is replaced by PTPA (Hombauer et al., 2007). At this point, PTPA catalyzes the activation of PP2A-C, altering the conformation of the active site and allowing the proper coordination two $Mn^{2+}$ ions in the C subunit active site (Leulliot et al., 2006). After activation, PTPA is released from the AC core dimer, which is then free to interact with PPM1. PPM1 is required for full PP2A activity, but methylation of PP2A-C is not a prerequisite for formation of all holoenzymes. Only PP2A-B B family members require PP2A-C methylation for complex formation *in vitro* and *in vivo.* However, loss of PPM1 function causes a disproportionate decrease in overall PP2A activity (Hombauer et al., 2007). After interacting with PPM1, the AC core dimer interacts with regulatory B subunits to form functional heterotrimeric PP2A holoenzymes.

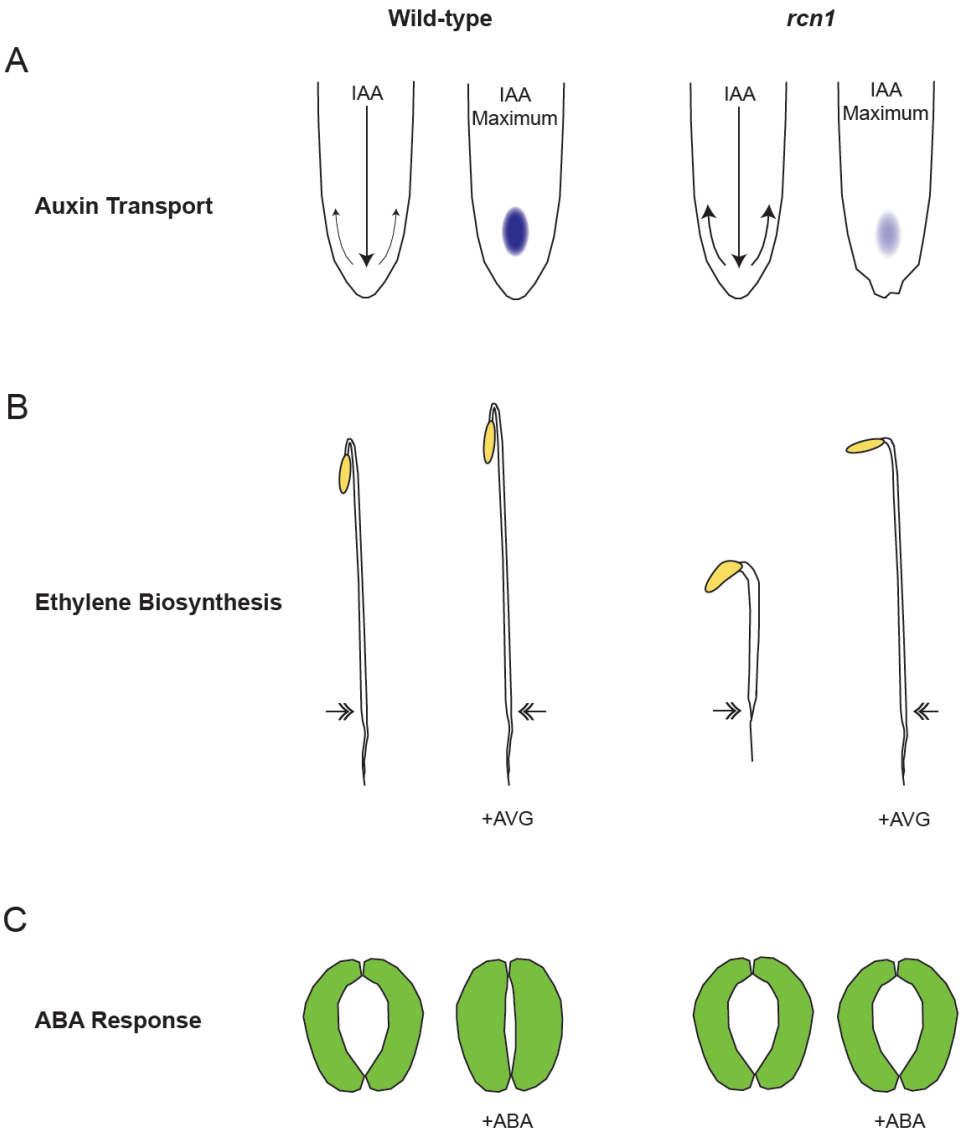



**Figure 5. Characteristic *rcn1* hormonal defects.** (A) Wild-type seedlings transport auxin from the shoot apical meristem towards the root (left panel). The auxin stream transport is then redirected back up towards the root-shoot junction, a process known as basipetal auxin transport. The regulated transport of auxin leads to the creation of a local concentration maximum near the root tip (shown in blue), which is important for directing root biological processes. *rcn1* roots have increased basipetal auxin transport, which depletes the characteristic auxin maximum found in wild-type roots (right). The perturbation of auxin-mediated developmental cues leads to root dysfunction and altered morphology (Blakeslee et al., 2008; Rashotte et al., 2001). (B) Wild-type seedlings produce very low levels of ethylene when grown in the dark, which allows hypocotyl elongation (left). When wild-type seedlings are treated with the ethylene biosynthesis inhibitor, AVG, they show slightly increased hypocotyl elongation over non-

treated plants. The *rcn1* mutant overproduces ethylene, leading to a short hypocotyl phenotype (right). This phenotype is rescued when *rcn1* seedlings are grown on AVG. (C) Wild-type plants close their stomata in response to the hormone ABA, which is a hormonal transducer of drought stress (left). In contrast, *rcn1* plants show reduced ABA sensitivity and fail to close their stomata upon application of ABA (right; Kwak et al., 2002).

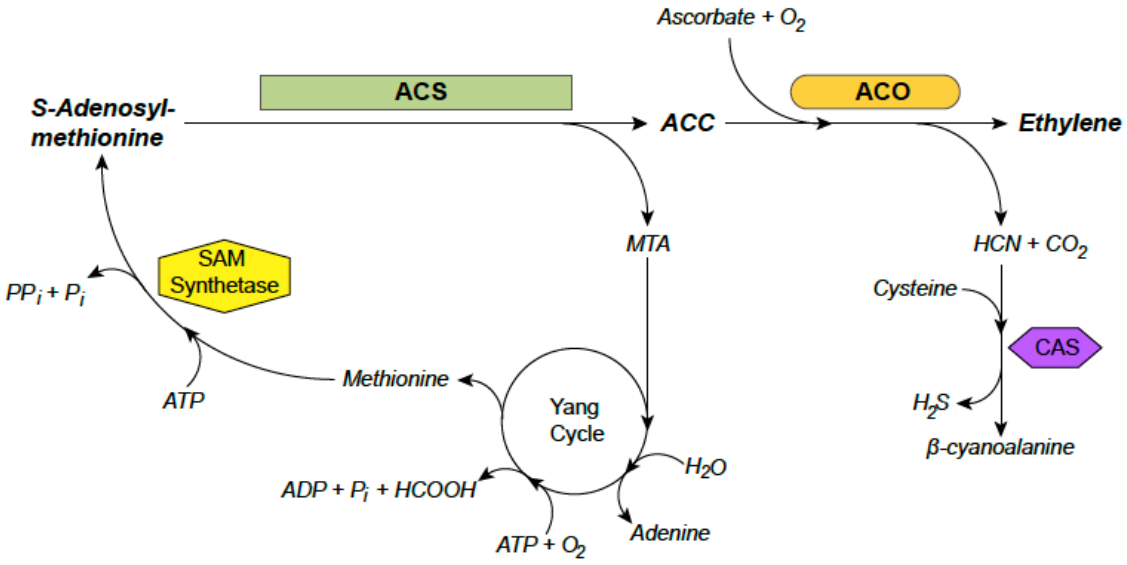
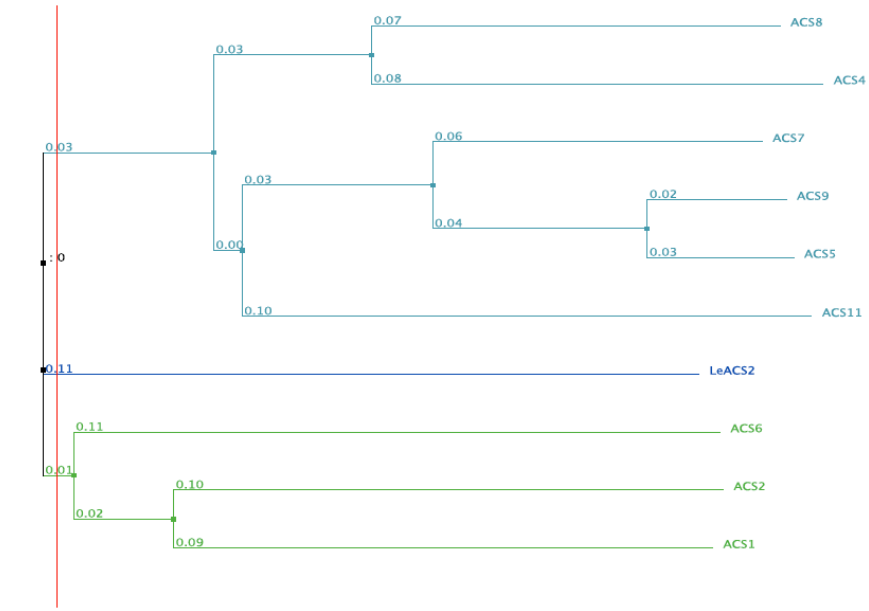



**Figure 6. The core ethylene biosynthetic pathway and associated metabolic circuitry.** Methionine is converted to S-adenosyl-methionine (SAM) by the action of SAM synthetase. SAM functions as a common precursor in many metabolic pathways. In the first committed step in ethylene biosynthesis, SAM is converted to 1-aminocyclopropane carboxylate (ACC) by the action of a family of ACC synthase (ACS) enzymes. A byproduct of this reaction is 5'methylthioadenosine (MTA). MTA is shunted into the Yang Cycle, where a series enzymatic reactions salvages methionine. The final step in ethylene biosynthesis is catalyzed by ACC oxidase (ACO), which cleaves ACC to form ethylene, cyanide, and $CO_2$. Cyanide is detoxified by β-cyanoalanine synthase (CAS). Enzymes are represented as colored geometric shapes. Metabolic substrates, intermediates, and products are italicized. The primary components of the ethylene biosynthetic pathway are in bold.

A


0.07
ACS8
0.03
0.08
ACS4
0.06
ACS7
0.03
0.03
0.02
ACS9
0.04
0.00
0.03
ACS5
0
0.10
ACS11
0.11
LeACS2
0.11
ACS6
0.01
0.10
ACS2
0.02
0.09
ACS1

B

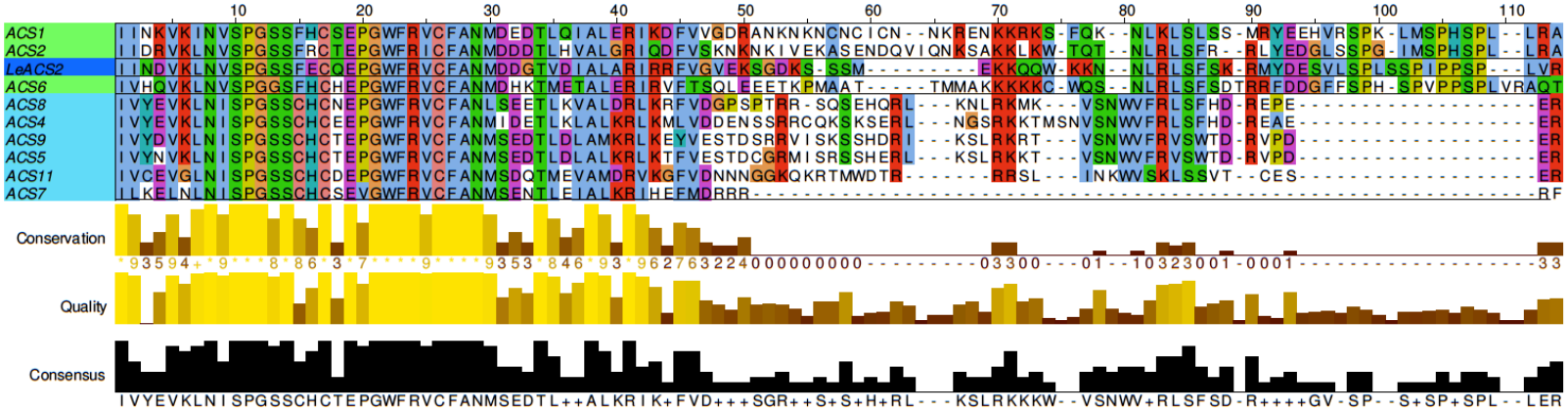

10
20
30
40
50
60
70
80
90
100
110
ACS1
ACS2
LeACS2
ACS6
ACS8
ACS4
ACS9
ACS5
ACS11
ACS7
Conservation
Quality
Consensus

**Figure 7. Arabidopsis ACS isozymes group into two phylogenetic clades.** (A) A phylogenetic tree constructed using the C-terminal sequences of all Arabidopsis ACS isozymes as well as LeACS2. The entire protein sequence for all ACS isozymes was first aligned using the MAFFIT software package. The C-terminal sequences, from amino acid position 400 on, using LeACS2 as the benchmark, were then selected and realigned using the MAFFIT software package and visualized using MSA. Arabidopsis ACS isozymes cluster into two distinct phylogenetic clades containing ACS8, 4, 7, 9, 5, and 11 and ACS6, 2, and 1. (B) The C-terminal sequence alignment that corresponds to the tree shown in (A). ACS isozyme names are color coded to match the tree shown in (A).

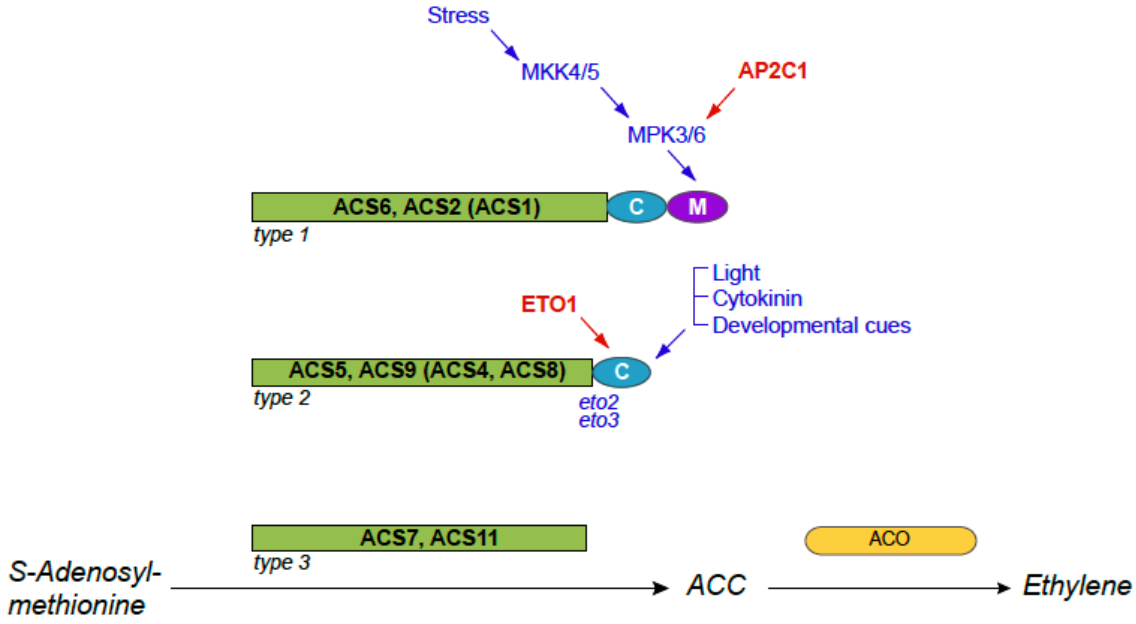



**Figure 8. Posttranslational modifications of ACS proteins regulate ethylene synthesis.** The three ACS isozyme types are defined by their C-terminal amino acid sequence motifs; representative members of each type are shown (Yoshida et al., 2006). Putative consensus sites for MAPK and CDPK phosphorylation are shown as purple and turquoise ovals, respectively. Several factors and signals that affect the stability of type 1 and type 2 isozymes through action on C-terminal motifs are indicated, with positive regulators shown in blue and negative regulators shown in red. In the absence of a stabilizing input, type 1 and type 2 isozymes are rapidly degraded by the 26S proteasome. Evidence for CDPK regulation of type 1 isozymes has been obtained in tomato fruits but has not yet been established in Arabidopsis. Although the C-terminus of type 2 isozymes can be phosphorylated by CDPKs *in vitro*, a regulatory role for CDPK phosphorylation of these proteins has not been demonstrated *in vivo*. The *eto2* and *eto3* mutations are stabilizing alleles of ACS5 and ACS9, respectively.

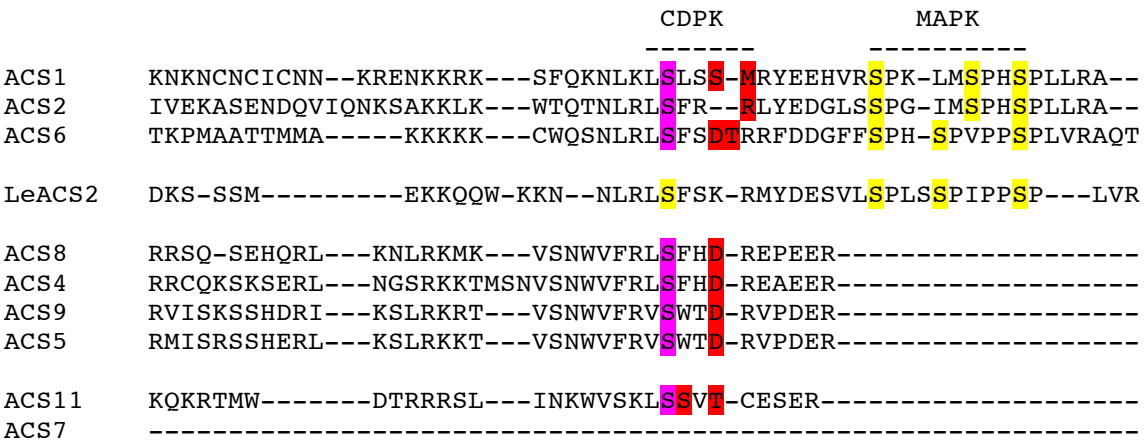



**Figure 9. Serine phosphorylation sites in tomato and Arabidopsis ACS isozymes.** The three C-terminal MAPK phosphorylation sites for ACS1, 2, and 6 as well as LeACS2 are highlighted in yellow, indicating that they are confirmed phosphorylation targets. Each of these three serine phosphorylation sites is immediately followed by a proline, which is expected for a MAPK target site. The upstream CDPK phosphorylation motif is highlighted in yellow for LeACS2, since it has been established as an *in vivo* phosphorylation target (Tatsuki and Mori 2001). The consensus sequence associated with CDPK phosphorylation is (hydrophobic$_{-1}$ – (serine/threonine target) – hydrophobic$_{+1}$ – any residue$_{+2}$ – basic$_{+3}$ – basic$_{+4}$; Hernandez-Sebastia et al., 2004). The putative CDPK target serine has been highlighted in purple, while surrounding residues that deviate from the defined consensus are shown in red. Type 1 ACS isozymes (1, 2, and 6) all posses the serine residue, but deviate from the surrounding consensus by 1-2 residues, disfavoring them as putative CDPK substrates in Arabidopsis. ACS11 also deviates from the CDPK consensus by 2 residues, indicating that it is probably not a substrate *in vivo.* The type 2 ACS isozymes (8, 4, 9, and 5) all deviate in the basic$_{+3}$ residue, but this is the only deviation from the consensus. The *in vivo* relevance of CDPK phosphorylation on type 2 enzymes requires further study. The presence and absence of phosphorylation target motifs in ACS isozymes is summarized in Table 3.

**Table 1. PP2A aliases**

| | Protein Family | Gene Name | Aliases |
|---|---|---|---|
| **Arabidopsis** | PP2A-A | RCN1 | At1g25490 - EER1, A1, PP2AA1 |
| | | PP2AA2 | At3g25800 - A2, PDF1 |
| | | PP2AA3 | At1g13320 - A3, PDF2 |
| | PP2A-B B | PP2AB1 | At1g51690 - B-alpha |
| | | PP2AB2 | At1g17720 - B-beta |
| | PP2A-B B' | PP2AB3 | At5g03470 - B'-alpha |
| | | PP2AB4 | At3g09880 - B'-beta |
| | | PP2AB4 | At4g15415 - B'-gamma |
| | | PP2AB6 | At3g26030 - B'-delta |
| | | PP2AB7 | At3g54930 - B'-epsilon |
| | | PP2AB8 | At3g21650 - B'-zeta |
| | | PP2AB9 | At3g26020 - B'-eta |
| | | PP2AB10 | At1g13460 - B'-theta |
| | | PP2AB11 | At5g25510 - B'-gamma-like |
| | PP2A-B B" | PP2AB12 | At5g18580 - FASS, TON2, GORDO, EMB40 |
| | | PP2AB13 | At5g44090 - B"-alpha |
| | | PP2AB14 | At1g03960 - B"-beta |
| | | PP2AB15 | At1g54450 - B"-gamma |
| | | PP2AB16 | At5g28850 - B"-delta |
| | | PP2AB17 | At5g28900 - B"-epsilon |
| | PP2A-C | PP2AC1 | At1g59830 - C1 |
| | | PP2AC2 | At1g10430 - C2 |
| | | PP2AC3 | At2g42500 - C3 |
| | | PP2AC4 | At3g58500 - C4 |
| | | PP2AC5 | At1g69960 - C5 |
| | PTPA | PTPA | At4g08960 - PTPA |
| | PPM1 | PPM1 | At1g02100 - PPM1, LCMT |
| | PME1 | PME1 | At4g10050 - PME1 |
| | | | |
| **Yeast** | PP2A-A | TPD3 | |
| | PP2A-B B | CDC55 | |
| | PP2A-B B' | RTS1 | |
| | PP2A-C | PPH21 | |
| | | PPH22 | |
| | PTPA | RRD1 | |
| | | RRD2 | |
| | PPM1 | PPM1 | |
| | PME1 | PPE1 | |

| | | | |
|---|---|---|---|
| **Human** | PP2A-A | PPP2R1A | A-alpha, PR65-alpha |
| | | PPP2R1B | A-beta, PR65-beta |
| | PP2A-B B | PPP2R2A | B-alpha, PR55-alpha |
| | | PPP2R2B | B-beta, PR55-beta |
| | | PPP2R2C | B-gamma, PR55-gamma |
| | | PPP2R2D | B-delta, PR55-delta |
| | PP2A-B B' | PPP2R5A | B'-alpha, PR61-alpha, B56-alpha |
| | | PPP2R5B | B'-beta, PR61-beta, B56-beta |
| | | PPP2R5C | B'-gamma, PR61-gamma, B56-gamma |
| | | PPP2R5D | B'-delta, PR61-delta, B65-delta |
| | | PPP2R5E | B'-epsilon, PR61-epislon, B56-epsilon |
| | PP2A-B B" | PPP2R3A | B"-alpha, PR130, PR72 |
| | | PPP2R3B | B"-beta, PR70 |
| | | PPP2R3C | B"-delta, G5PR |
| | PP2A-C | PPP2CA | PP2A-C-alpha |
| | | PPP2CB | PP2A-C-beta |
| | PTPA | | PTPA |
| | PPM1 | | LCMT-1 |
| | PME1 | | PME-1 |

**Table 2. Properties used to distinguish PPP family protein phosphatases.**

| | PP1 | PP2A | PP2B | PP2C |
|---|---|---|---|---|
| Preference for the α or β subunit of phosphorylase kinase | β | α | α | α |
| Phosphorylase phosphatase activity | Very low | High | Very low | High |
| Requirement for divalent cations | NO | NO | YES ($Ca^{2+}$) | YES ($Mg^{2+}$) |
| **Inhibition by okadaic acid** | **YES ($IC_{50}$ = 10nM)** | **YES ($IC_{50}$ = 0.1nM)** | **YES (weak)** | **NO** |
| **Inhibition by cantharidin** | **YES (mam. $IC_{50}$ = 473nM) (plant $IC_{50}$ = 60nM)** | **YES ($IC_{50}$ = 40nM)** | **YES (weak)** | **NO** |
| Inhibition by microcystin-LR | YES ($IC_{50}$ = 0.1nM) | YES ($IC_{50}$ = 0.1nM) | YES (weak) | NO |

Note normally PP1 and PP2A bind $Mn^{2+}$ very tightly and are therefore not dependent on cations in assay conditions. Table is modified from (MacKintosh et al., 1990; Stubbs et al., 2001).

**Table 3. Summary of ACS phosphorylation target motifs and interacting proteins.**

| Isozyme | Type[a] | CDPK site[b] | MAPK site[c] | 14-3-3Ω Client[d] | TOE sequence[e] | Xbat32 Interactor[f] |
|---|---|---|---|---|---|---|
| **ACS1** | 1 | No | YES | - | No | - |
| **ACS2** | 1 | No | YES | - | No | No |
| **ACS6** | 1 | No | YES | YES | No | No |
| **ACS4** | 2 | YES | No | - | YES | YES |
| **ACS5** | 2 | YES | No | - | YES | - |
| **ACS8** | 2 | YES | No | YES | YES | - |
| **ACS9** | 2 | YES | No | - | YES | - |
| **ACS7** | 3 | No | No | YES | No | YES |
| **ACS11** | 3 | No | No | - | No | No |

Data is modified from a (Yoshida et al., 2006), b (Hernandez-Sebastia et al., 2004), c (Liu and Zhang, 2004), d (Chang et al., 2009), e (Yoshida et al., 2006), and f (Prasad et al., 2010). A – indicates that interaction has not been tested.

# CHAPTER 2: SPECIFICITY OF RCN1-MEDIATED PROTEIN PHOSPHATASE 2A REGULATION IN MERISTEM ORGANIZATION AND STRESS RESPONSE IN ROOTS

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*Plant Physiology,* February 2008, Vol. 146, pp. 539-553.

I was responsible for analysis of hypocotyl length in the *rcn1* complementation experiments shown in Figure 2, and for the analysis of protein expression levels shown in Figure S3. I participated in the analysis of root tip architecture defects using confocal microscopy shown in Figures 3, 5, and S4. Additionally, I contributed substantially to generation, selection, molecular characterization, and propagation of the transgenic lines used in this work.

**ABSTRACT**

Protein dephosphorylation by the serine/threonine protein phosphatase 2A (PP2A) modulates a broad array of cellular functions. PP2A normally acts as a heterotrimeric holoenzyme complex comprising a catalytic subunit bound by regulatory A and B subunits. Characterization of the regulatory A subunit isoforms (ROOTS CURL IN NAPHTHYLPHTHALAMIC ACID1 [RCN1], PP2AA2 and PP2AA3) of *Arabidopsis thaliana* PP2A has shown that RCN1 plays a primary role in controlling root and hypocotyl PP2A activity in seedlings. Here we show that hypocotyl and root growth exhibit different requirements for RCN1-mediated regulation of protein phosphatase 2A activity. Roots of *rcn1* mutant seedlings exhibit characteristic abnormalities in cell division patterns at the root apical meristem, as well as reduced growth under ionic, osmotic and oxidative stress conditions. We constructed chimeric A subunit genes and found that restoration of normal root tip development in *rcn1* plants requires both regulatory and coding sequences of *RCN1*, while the hypocotyl elongation defect of *rcn1* plants can be complemented by either *RCN1* or *PP2AA3* transgenes. Furthermore, the *RCN1* and *PP2AA3* proteins exhibit ubiquitous subcellular localization patterns in seedlings and both associate with membrane compartments. Together, these results show that RCN1-containing PP2A has unique functions that cannot be attributed to isoform-specific expression and localization patterns. Post-embryonic *RCN1* function is required to maintain normal auxin distribution and stem cell function at the root apex. Our data show

that *RCN1*-regulated phosphatase activity plays a unique role in regulating post-embryonic root development and stress response.

## INTRODUCTION

Regulated dephosphorylation by protein phosphatases has emerged as a universal control mechanism in physiology and development. Important roles have been identified for a variety of protein phosphatase (PP) species in plants. For instance, several PP2C enzymes negatively regulate abscisic acid response (reviewed in Schweighofer et al., 2004; Yoshida et al., 2006), while other PP2Cs modulate wound signaling, stress signaling and meristem development (Stone et al., 1998; Song and Clark, 2005; Schweighofer et al., 2007). Similarly, distinct dual-specificity phosphatases regulate carbohydrate metabolism (Kerk et al., 2006; Niittyla et al., 2006; Sokolov et al., 2006) and oxidative, saline and genotoxic stress tolerance (Ulm et al., 2002; Lee and Ellis, 2007). The serine/threonine protein phosphatase 2A (PP2A) constitutes an abundant population of oligomeric enzymes that play crucial roles in the regulation of growth and development. Altered PP2A activity in plants has been linked to defects in hormone homeostasis and signaling, defense responses, cell division, morphogenesis and reproduction (reviewed in DeLong, 2006). Analysis of Arabidopsis PP2A mutants suggests that important substrates for PP2A include proteins that control microtubule dynamics (Camilleri et al., 2002) and components of the auxin transport apparatus (Rashotte et al., 2001; Shin et al., 2005; Michniewicz et al., 2007).

The predominant form of PP2A is a heterotrimeric complex containing a catalytic (C) subunit, a scaffolding/regulatory (A) subunit and a regulatory (B) subunit (Janssens and Goris, 2001). Combinatorial diversity of these heterotrimers enhances the versatility of the enzyme complex. The C and A subunits are abundant, ubiquitous and highly conserved, while B subunits exhibit more specific expression patterns and are encoded by several unrelated gene families. Localization and substrate specificity of PP2A action are controlled largely through the effects of bound A and B subunits. The regulatory A subunit comprises 15 imperfect repeats of the alpha-helical HEAT (Huntingtin, Elongation factor 3, A subunit, and TOR proteins; (Andrade and Bork, 1995). The carboxy-terminal repeats bind the catalytic subunit, and the amino-terminal repeats bind the B subunit. Both binding interactions employ a hydrophobic binding interface formed by short and variable loops located in the center of each HEAT repeat (see Supplemental Figure S1A; Ruediger et al., 1994; Xing et al., 2006). The A subunit performs at least three crucial regulatory functions. First, binding of the A subunit alters the kinetic properties of the C subunit (Price and Mumby, 2000). Second, A subunit binding also allows interaction of C subunits with the diverse B subunits involved in targeting PP2A function to its physiological targets (Ruediger et al., 1994). Third, recent work indicates that A subunit binding is required for acquisition of the fully activated C subunit conformation (Hombauer et al., 2007). A single regulatory A subunit isoform appears to suffice in rice and maize, as well as in several fungi (van Zyl et al., 1992; Kinoshita et al., 1996; Yu et al., 2001),

while mammalian systems rely on two differentially expressed and functionally distinct isoforms (Zhou et al., 2003; Sablina et al., 2007).

The Arabidopsis genome encodes three functionally distinct A subunit isoforms (RCN1, PP2AA2 and PP2AA3; Slabas et al., 1994; Zhou et al., 2004), with RCN1 alone acting as a key positive regulator of PP2A activity in seedlings. Biochemical and physiological analyses show reduced PP2A enzymatic activity in *rcn1* plants, and *rcn1* mutant phenotypes result from loss of PP2A activity in vivo (Deruère et al., 1999; Rashotte et al., 2001; Kwak et al., 2002; Larsen and Cancel, 2003). Basipetal auxin transport is increased in hypocotyls and roots of *rcn1* seedlings, resulting in altered gravitropic response in both organs (Rashotte et al., 2001; Shin et al., 2005; Muday et al., 2006). *RCN1* also functions as a transducer of ABA signals, acting upstream of ABA-induced increases in cytosolic $Ca^{2+}$, but downstream of the PP2C ABI1 (Kwak et al., 2002), and as a negative regulator of ethylene synthesis (Larsen and Chang, 2001; Muday et al., 2006). Additional data suggest a negative regulatory role for *RCN1* in ethylene signaling in shoots (Larsen and Chang, 2001; Larsen and Cancel, 2003).

The abnormal phenotypes of *rcn1* mutant plants show that RCN1-containing PP2A species perform regulatory functions that are not mediated by complexes containing the other regulatory A subunit isoforms. Despite gene expression patterns that overlap with that of *RCN1*, loss of *PP2AA2* and/or *PP2AA3* function does not significantly alter phosphatase inhibitor sensitivity or produce dramatic

mutant phenotypes (Zhou et al., 2004). However, plants carrying a *pp2aa2* or *pp2aa3* mutation in combination with *rcn1* exhibit severe morphological and developmental abnormalities including arrested primary root growth (Zhou et al., 2004; Michniewicz et al., 2007). Degeneration of the primary root apical meristem in *rcn1 pp2aa2* and *rcn1 pp2aa3* seedlings appears to be caused by loss of auxin signaling, and is associated with relaxed or reversed localization of PIN-FORMED (PIN) proteins in embryos and seedling roots (Michniewicz et al., 2007). The radial cell and organ expansion phenotypes exhibited by these seedlings are similar to those of seedlings grown in the presence of high doses of phosphatase inhibitors, and therefore demonstrate the effect of drastic loss of PP2A activity (Rashotte et al., 2001; Shin et al., 2005; Muday et al., 2006).

We asked whether we could distinguish between functions that specifically require the RCN1 protein sequence and functions that are sensitive to overall A subunit dosage but insensitive to isoform specificity. To address this question, we undertook functional analyses in vivo, using constructs that carry regulatory and coding sequences from different A subunit-encoding genes to rescue *rcn1* defects in seedling hypocotyls and in roots. The reduced hypocotyl elongation phenotype of *rcn1* can be rescued by *PP2AA3* constructs; however, restoration of wild-type root tip organization requires both promoter and coding sequences of *RCN1*. Thus hypocotyl growth is sensitive to A subunit gene dosage but relatively insensitive to isoform specificity, while regulation of root growth requires PP2A complexes containing the RCN1 regulatory subunit. We show that loss of *rcn1*

alone causes increased sensitivity to a broad panel of stress treatments and compromises the maintenance of organized stem cell populations. Inhibition of phosphatase activity in seedlings is sufficient to recapitulate the *rcn1* phenotype at the root apex, indicating that RCN1-mediated phosphatase regulation is required post-embryonically for normal meristem function. Our data suggest that the RCN1 protein specifically mediates interactions targeting PP2A to substrates required for root stress response and meristem function.

## RESULTS

### Arabidopsis RCN1-YFP fusions retain biological activity

To facilitate our investigation of the biological specificity determinants for *RCN1* function in plants, we constructed amino- and carboxy-terminal fusions of *RCN1* with the YFP reporter (see Materials and Methods). We first tested cDNA fusions for biological activity using a complementation assay in the yeast PP2A regulatory A subunit mutant, *tpd3-1*. The *tpd3-1* mutation confers temperature sensitivity and slow growth phenotypes that are complemented by the *RCN1* cDNA (Garbers et al., 1996), as well as sensitivity to stress conditions such as nitrogen starvation and osmotic stress (Santhanam et al., 2004). RCN1-YFP amino- and carboxy-terminal fusion proteins were expressed under control of the constitutive alcohol dehydrogenase promoter (Ammerer, 1983). Both constructs rescued growth of *tpd3* mutant at high temperature, indicating that the fusion proteins are competent to regulate the yeast PP2A complex (Figure 1A). YFP fluorescence was detected in yeast cells carrying *RCN-YFP* and *YFP-RCN* (Figure 1B) and full-length fusion proteins were detected by anti-GFP (Figure 1C) and anti-RCN1 (see Supplemental Figure S1B) antibodies. Although the predicted molecular weights of the two fusion proteins were nearly identical (94 kDa), the RCN1-YFP fusion consistently exhibited a slightly slower SDS-PAGE migration when extracted from both yeast and plant cells (see below). The *RCN1-YFP* fusion also complemented the *tpd3* stress sensitivity phenotype and rescued growth in the presence of salt (Figure 1D) and sorbitol (data not shown).

Thus fusion of YFP to either terminus does not impair the regulatory A subunit function of RCN1 in yeast.

**YFP-RCN1 and YFP-PP2AA3 fusions rescue the rcn1 hypocotyl elongation defect**

To assay the biological activities of RCN1-YFP fusions in planta, we used a TT-PCR template overlap strategy (Tian et al., 2004) to generate translational fusions in the context of the full-length genomic *RCN1* sequence (Figure 2A). The resulting constructs carried 2.1 kb of genomic sequence upstream from the RCN1 transcript, the transcribed region (including introns) and 550 bp of downstream sequence. Both amino- and carboxy- terminal fusions were generated in the plant transformation vector pPZP221 (Hajdukiewicz et al., 1994) and transformed into *rcn1-1* mutant plants. To test for biological function, we assayed for complementation of the *rcn1* hypocotyl elongation defect (Figure 2B). In families segregating for an $RCN_{pro}$*:YFP-RCN1* (*RYR*) or $RCN_{pro}$*:RCN1-YFP* (*RRY*) fusion, YFP fluorescence segregated with rescued hypocotyl elongation, while hypocotyl lengths of YFP-negative siblings matched those of the *rcn1* parent and the empty vector control. These data indicate that the YFP fusion proteins provide A subunit function in plants, as well as in yeast.

To allow direct comparison of the localization and biological activities of the RCN1 and PP2AA3 isoforms, we constructed equivalent YFP fusions to the *PP2AA3* cDNA (Figure 2A). In one derivative the *YFP-PP2AA3* fusion remained

under control of the *RCN1* promoter and 5' UTR (*RYA*), while in the second construct the *RCN1* upstream sequences were replaced with a 1.2 kbp fragment containing the *PP2AA3* leader (including a 312 bp intron), the intragenic region and the predicted 5' end of the next gene upstream (*AYA*). These constructs were designed to drive expression of the *YFP-PP2AA3* fusion in the *RCN1* and the *PP2AA3* domains, respectively. To avoid overexpression artifacts, we focused primarily on transformants carrying single copy T-DNAs in the *rcn1* background. We assayed the ability of these fusions to complement the hypocotyl elongation defect of *rcn1* seedlings (Figure 2C). Hypocotyl growth was fully restored in lines carrying the *YFP-PP2AA3* fusion under control of either the *RCN1* or *PP2AA3* promoter. Only a slight difference was observed between lines expressing *YFP-RCN* vs. *YFP-PP2AA3* fusions, and this difference was eliminated in lines carrying *YFP-PP2AA3* in two or more copies (Figure 2C, lines *rcn1 RYA-32*, *rcn1 AYA-6* and *rcn1 AYA-16*). These data indicate that A subunit dosage is critical for supporting normal hypocotyl elongation, but RCN1-specific protein sequences are not strictly required for this activity.

**RCN1-specific PP2A regulation maintains root tip organization**

We used confocal imaging to determine whether *rcn1* seedlings exhibit root tip disorganization phenotypes consistent with our hypothesis that increased basipetal auxin transport alters auxin distribution in *rcn1* roots. The normal root apical meristem exhibits a stereotypical arrangement of initial cells organized around a small population of quiescent center (QC) cells (reviewed in (Benfey

and Scheres, 2000), and root tip architecture is disrupted by factors that alter the position or magnitude of an auxin concentration maximum in the root apex (Sabatini et al., 1999). While wild-type roots maintained highly regular cell numbers and arrangement in the quiescent center, columella initials and root cap columella, *rcn1* seedlings showed disorganization indicating aberrant cell division patterns (Figure 3). Quiescent center (QC) and cortical/endodermal initial cells were difficult to distinguish in *rcn1* seedlings and frequently failed to form a well-defined layer at the bottom of the stele. Columella cell files were abnormal in 68% of *rcn1* roots examined (versus 12% of wild-type roots), while columellar tiers were disrupted in 28% (versus 0 in wild-type; n = 25 for each genotype). In *rcn1* roots, cells formed irregular layers and cell numbers varied between different tiers. In several roots with three files of proper columella cells, a flanking file of lateral root cap cells appeared to have been recruited into a 'shoulder' of the columella. All wild-type roots scored as abnormal exhibited regular files and tiers but had an abnormal number of cell files. As shown below, an independent *rcn1* allele in the Columbia genetic background showed similar defects in root tip organization.

We asked whether *YFP-RCN* and *YFP-PP2AA3* could rescue the abnormal morphology of *rcn1* root tips (Figure 3). *RRY* and *RYR* lines exhibited identifiable QCs and clear restoration of columellar cell organization. In a representative RYR line, only 7% of roots exhibited an abnormal number of columellar files (n = 15). In contrast, columellar file number remained abnormal in roots of a

representative *RYA* line (35%; n = 23) and a representative *AYA* line (26%; n = 31). Similarly, a regular columella initial cell layer was present in all wild-type and *RYR* root tips, with only 7% exhibiting an abnormal columella initial cell number, while 53% of *rcn1-1* roots, 30% of *RYA* roots and 35% of *AYA* roots exhibited abnormal cell numbers in a columella initial cell layer that frequently was poorly defined. These data suggest that normal root tip organization specifically requires *RCN1* function; *YFP-PP2AA3* constructs that support normal hypocotyl elongation do not fully restore normal root growth. To ensure that fusion to YFP did not impair function of PP2AA3 protein in roots, we also assayed for rescue by a native *PP2AA3* construct (*$PP2AA3_{pro}$:PP2AA3*). The native *PP2AA3* construct also provided only weak complementation of *rcn1* root tip defects (data not shown). These data show that the requirements for A subunit function are more stringent in the root tip than in the hypocotyl, and suggest that increased *PP2AA3* dosage does not completely compensate for loss of *RCN1* function in the root tip.

Our earlier work on A subunit double mutants revealed severe root growth defects in *rcn1 pp2aa2* and *rcn1 pp2aa3* double mutants, but not in *pp2aa2 pp2aa3* double mutant seedlings (Zhou et al., 2004). We asked whether root growth in the *pp2aa2 pp2aa3* double mutant background was sensitive to decreased *RCN1* dosage. We assessed root tip morphology by visualizing amyloplasts in cleared root tips after staining for starch accumulation. As expected, starch staining revealed a highly regular arrangement of columellar

cells in wild-type and *pp2aa2 pp2aa3* root tips, with clearly defined tiers and files of cells (see Supplemental Figure S2). In *rcn1* root tips, columellar cell files and tiers often were irregular, indicating aberrant patterns of columella initial divisions. Overall columellar morphology in the *pp2aa2 pp2aa3 rcn1*/+ mutant was similar to that observed in the *rcn1* background, with most roots exhibiting four poorly defined tiers of cells, and many lacking part or all of one columellar cell file. Consistent with a recent report, columellar cell numbers were severely reduced and columellar files and tiers were difficult to identify in *rcn1 pp2aa2* and *rcn1 pp2aa3* root tips even at 4 d.p.g. (see Supplemental Figure S2; Michniewicz et al., 2007). We conclude that *RCN1* function is required for normal root tip organization, and root growth is sensitive to *RCN1* dosage. Although *RCN1* becomes haploinsufficient in the *pp2aa2 pp2aa3* background, the heterozygous *RCN1* dose in *pp2aa2 pp2aa3 rcn1*/+ mutants supports more normal development than a homozygous *PP2AA3* dose in the *rcn1 pp2aa2* double mutant.

**Normal root development requires post-embryonic RCN1 function**

Abnormal embryogenesis in *rcn1 pp2aa2* and *rcn1 pp2aa3* plants (Zhou et al., 2004; Michniewicz et al., 2007) as well as enhancement of *pin1* and *pid* embryogenesis defects by *rcn1* (Zhou et al., 2004) indicate that *RCN1* is required for normal embryo development. To determine whether the root tip disorganization phenotype reflects an embryonic or a post-embryonic requirement for RCN1 action, we asked whether chemical inhibition of protein

phosphatase activity in seedling roots was sufficient to produce a phenocopy of *rcn1* root tip defects. We and others have previously used cantharidin to produce a phenocopy of *rcn1* in organ elongation, root curling and basipetal auxin transport assays (Deruère et al., 1999; Rashotte et al., 2001) and other phosphatase inhibitors have been used to mimic the ethylene and ABA response phenotypes of *rcn1* (Kwak et al., 2002; Larsen and Cancel, 2003). We compared the root apex phenotypes of wild-type (Col) seedlings grown in the absence or presence of cantharidin with those of seedlings carrying *rcn1-6*, a T-DNA insertion allele (see Materials and Methods). RCN1 protein is undetectable in extracts of *rcn1-6* seedlings, and the gross hypocotyl and root phenotypes of *rcn1-6* seedlings are indistinguishable from those of the *rcn1-1* allele (data not shown). Poorly defined quiescent centers and irregular columellar cell arrangements were observed in 86% of cantharidin-treated wild-type roots (n = 24), closely matching the defects exhibited by 94% of *rcn1-6* seedlings grown in the absence of inhibitor (n = 18; Figure 4A - D). Similar results were obtained with cantharidin-treated wild-type roots of another accession (Ws; data not shown). Thus the *rcn1* phenotype reflects a post-embryonic requirement for normal PP2A regulation, as protein phosphatase inhibition in seedlings is sufficient to disrupt normal root tip development.

The disrupted columella organization observed in *rcn1* roots suggests abnormal function of the stem cell populations, particularly the columella initials and/or QC. We asked whether loss of phosphatase activity altered the expression of a

molecular marker for QC identity. In roots the expression of a GFP reporter fused to the *AGL42* MADS-box gene is tightly restricted to the QC at 4 d.p.g. (Nawy et al., 2005). Normal root tip organization and clear QC expression of *AGL42-GFP* were observed in 87% of untreated roots (n = 23; Figure 4E). Strongly reduced expression of *AGL42-GFP* and abnormal root tip architecture were observed in 91% of cantharidin-treated seedling roots (n = 23; Figure 4F, G). In most cantharidin-treated roots, *AGL42-GFP* expression was reduced to background levels (Figure 4F - H). These observations are consistent with the hypothesis that loss of RCN1-regulated phosphatase activity compromises QC function. Reduced *AGL42-GFP* expression might be a consequence of decreased auxin accumulation at the root apex; however, we do not observe altered AGL42-GFP expression in auxin-treated root tips (data not shown). Furthermore, publicly available microarray data indicate that *AGL42* expression changes less than 1.5-fold in response to IAA or NAA treatment (AtGenExpress Hormone Response and Genvestigator Stimulus data sets).

We asked whether the activity of an auxin responsive reporter construct was altered in the *rcn1* mutant. As described previously (Sabatini et al., 1999), wild-type seedlings carrying the *DR5-GUS* reporter show most intense staining around the columella initials, with lower GUS activity levels in the QC and throughout the columella (Figure 4I). In *rcn1 DR5-GUS* roots, the overall intensity of staining was reduced, and *rcn1* roots frequently exhibited more intense staining in the external columella layer than in the region around the

columella initials (Figure 4J). Similar results were obtained with wild-type and *rcn1 DR5-GUS* lines backcrossed twice into the Ws genetic background (Figure 4K, L). Cantharidin-treated wild-type *DR5-GUS* seedlings also showed a reduction in staining intensity, as was reported previously (Shin et al., 2005) and data not shown). Reduced activity of a DR5rev reporter was recently reported for seedlings carrying a *pp2aa2* or *pp2aa3* mutation in combination with *rcn1* (Michniewicz et al., 2007). Our results indicate that even modest reductions in phosphatase activity reduce auxin concentrations around the initial cells and compromise meristem function. The ability of cantharidin treatment to mimic *rcn1* defects suggests that auxin distribution and QC identity are regulated by post-embryonic *RCN1* function.

**Abundance and localization of YFP fusion proteins**

We assayed the levels of transgene expression in roots via immunoblotting with anti-RCN1 and anti-GFP antibodies (Supplemental Figure S3). Anti-RCN1 immunoblots show that accumulation of the fusion proteins in the *RYR* and *RRY* lines was comparable to that of native RCN1 protein in wild-type plants. Use of anti-GFP antibodies allowed direct comparison of fusion protein abundance, which was similar in *RYA*, *RYR* and *RRY* lines, and somewhat greater than in *AYA* lines. These results show that intact fusion protein accumulates at levels comparable to those of the endogenous A subunits and indicate that complementation of the *rcn1* defects by *YFP-RCN1* does not require gross overexpression of the fusion protein.

We also assayed the expression and subcellular localization of the YFP fusions using confocal microscopy. In a recent study, immunolocalization of an RCN1-YFP fusion in cells of the root apex detected cytoplasmic, perinuclear and peripheral localization (Michniewicz et al., 2007). Consistent with this result, we observed that both RCN1-YFP fusion proteins were abundant and ubiquitous in root tips, with cytoplasmic and strong perinuclear signal accumulating in all cell types at the root apex (Figure 5A - F). Interestingly, although cells in the region of the root apical meristem (and distal elongation zone) exhibited very little nuclear RCN1-YFP signal, vacuolated cells in more mature regions of the root exhibited nuclear as well as cytoplasmic accumulation (Figure 5 D – F vs. G - I). Optical sectioning confirmed that the fusion protein was present inside the nucleus of these cells (data not shown). Cells in the apical meristem and in more mature regions also exhibited strong peripheral signal indicating enrichment around the plasma membrane; membrane enrichment was especially clear in cortical cells (Figure 5 G – L and Supplemental Figure S4A – C). Strong accumulation of fusion protein also was observed in root hairs and lateral root primordia, with localization in lateral root primordia recapitulating that observed at the primary root tip (data not shown). Light and dark-grown seedlings carrying the fusion constructs exhibited YFP fluorescence in roots and hypocotyls, with YFP accumulation patterns matching the previously reported RCN1 mRNA expression pattern (Deruère et al., 1999). Like vacuolated root cells, hypocotyl

cells exhibited cytoplasmic, nuclear and peripheral signal, with little or no accumulation evident in chloroplasts (data not shown).

To confirm that membrane localization was characteristic of the native forms of RCN1 and PP2AA3, we assayed the distribution of endogenous A subunits in soluble and microsomal membrane fractions isolated from wild-type seedlings (Figure 6A). All three A subunits were abundant both in soluble and microsomal fractions, consistent with the analysis of YFP-RCN1 fusion protein localization. The catalytic subunit also was detected in microsomal fractions (data not shown). In contrast to PP2A subunits, the cytosolic PEPC protein was detected in soluble but not membrane fractions, indicating minimal contamination of the membrane fraction with cytoplasmic proteins. We detected PP2AA2 and PP2AA3 in membranes extracted from *rcn1* mutant roots, and all three isoforms in the membrane fraction from wild-type roots (Figure 6B). These data indicate that PP2A complexes associate with membranes in growing seedlings. The presence of all three A subunit proteins in the microsomal fraction indicates that membrane association is not an isoform-specific characteristic. It is possible that recruitment of PP2A to a cellular membrane may allow it to interact with substrate or regulator proteins on the same membrane. Although the primary amino acid sequences of the A and C subunits do not contain motifs that predict membrane localization, several recent studies have shown that PP2A may interact with plasma membrane components, including the plasma membrane $H^+$-ATPase and the signaling lipid phosphatidic acid (Michniewicz et al., 2007).

Subcellular localization of the YFP-PP2AA3 fusion protein was similar to that of YFP-RCN1. Under control of the *RCN1* promoter, accumulation of the YFP-PP2AA3 fusion was similar to that of YFP-RCN1 (see Supplemental Figure S4D). Expression of YFP-PP2AA3 under control of the *PP2AA3* regulatory region resulted in decreased abundance of the fusion protein, with the strongest accumulation evident in the vascular cylinder (Figure 5M - O and Supplemental Figure S4E - F). At this lower abundance, subcellular localization patterns were difficult to assess, but perinuclear, cytoplasmic and peripheral localization were detected in most roots. These results suggest that RCN1 is more abundant than PP2AA3 in wild-type roots.

**RCN1 performs an isoform-specific function in root stress response**

Given the unique role of *RCN1* in regulating root growth and the stress sensitivity of PP2A mutants in yeast, we asked whether *rcn1* mutant plants show stress sensitivity similar to that observed in *tpd3* yeast cells. Roots of mutant seedlings exhibited increased sensitivity to ionic ($Na^+$, $K^+$), osmotic (mannitol) and oxidative (hydrogen peroxide) stress with decreased elongation across a range of concentrations (Figure 7A – D). Sodium and mannitol treatment also caused radial expansion, which was enhanced in the cortical cell layer of *rcn1* roots (Figure 7E and Supplemental Figure S5). Oxidative stress inhibited elongation (Figure 7D) but did not cause radial swelling of wild-type or mutant roots (data not shown). Both *YFP-RCN1* and *RCN1-YFP* fusion transgenes restored wild-

type stress tolerance to *rcn1* mutant roots (Figure 7F). These data indicate that RCN1 regulation of PP2A activity is required for normal stress tolerance in seedling roots. We did not detect differences in salt sensitivities of adult wild-type and *rcn1* plants, suggesting that the stress sensitivity of *rcn1* is limited to the seedling stage (data not shown).

Unlike *rcn1*, the *pp2aa2* and *pp2aa3* single mutants and the *pp2aa2 pp2aa3* double mutant exhibited stress sensitivities that very nearly matched that of the parental wild-type (see Supplemental Figure S6). Despite the presence of PP2AA2 and PP2AA3 proteins in root tissue (Zhou et al., 2004), these regulatory A subunit isoforms do not appear to play an equivalent role in stress tolerance. We asked whether the amino acid sequences for RCN1, PP2AA2 and PP2AA3 exhibit discrete differences that might confer biological specificity. The predicted RCN1 protein shares 86% identity with both PP2AA2 and PP2AA3 (see Supplemental Figure S1A; (Slabas et al., 1994), and most of the strongly conserved residues defined in mammalian A subunits are conserved in all three Arabidopsis isoforms. However, Tyr450, one putative component of the hydrophobic interaction interface (Ruediger et al., 1994; Groves et al., 1999; Xing et al., 2006), is replaced by a basic residue (His) in PP2AA2 and PP2AA3 (see Supplemental Figure S1A). Mutagenesis of the *RCN1-YFP* fusion to generate a His450 allele does not compromise complementation of temperature, sorbitol and sodium chloride sensitivity of *tpd3* yeast cells (data not shown), suggesting that Tyr450 does not play a required role in mediating stress tolerance. Intriguingly,

database searches suggest that the His450 A subunit may be a plant-specific variant. A Tyr residue is conserved at this position in A subunits of all vertebrates and insects, in *S. cerevisiae* and in many plant species (pea, tobacco, Brassica, Medicago and Lolium). Rice, maize, several other grasses, many fungi and *C. elegans* carry Phe at this position. His450 isoforms are found only in plant genomes which also encode a Tyr450 isoform (e.g. those of Vicia, Medicago, Brassica and Arabidopsis) and in *Thellungiella salsuginea*, for which limited sequence data are available. The His450 variant thus appears to be a plant-specific regulatory A subunit isoform, and may occur only in plants that also encode a Tyr450 isoform.

## DISCUSSION

Our analysis of Arabidopsis A subunit functions in vivo demonstrates unique functions for *RCN1* in specific cell types in seedlings. The requirement for *RCN1* function in maintaining normal root stem cell organization is not explained by cell- or tissue-specific mRNA expression patterns, since expression of *PP2AA3* under control of the *RCN1* promoter does not fully rescue normal cell division patterns. In hypocotyl tissue, rescue of normal cell expansion by *PP2AA3* expression demonstrates overlapping function of the A subunit isoforms. Our data support the hypothesis that root growth and stress response are regulated by PP2A substrates specifically targeted by RCN1, while targeting of substrates involved in hypocotyl growth is not dependent on a particular A subunit isoform. Organization and function of stem cells at the root apex, as measured by formation of normal columella tiers and expression of a marker for QC identity, is compromised by reduced PP2A function during post-embryonic development. Given that the subcellular localization of RCN1 and PP2AA3 proteins appears similar, functional specificity is likely to depend on isoform-specific protein-protein interactions with substrates or regulators of the complex.

### Dosage sensitivity vs. isoform specificity in A subunit function

*YFP-PP2AA3* fusion constructs that provide only weak complementation of the *rcn1* root tip phenotype robustly rescue hypocotyl elongation. These findings suggest that hypocotyl elongation requires a threshold level of A subunit function,

with no stringent requirement for RCN1-specific amino acid sequences. Even a modest level of YFP-PP2AA3 expression in the *rcn1* mutant is sufficient to promote normal hypocotyl elongation. Since increased ethylene synthesis in dark-grown *rcn1* seedlings contributes significantly to reduced hypocotyl growth (Larsen and Chang, 2001; Muday et al., 2006), this result suggests that PP2A complexes containing the PP2AA3 isoform are competent for down-regulation of ethylene biosynthesis. In contrast, *PP2AA3* rescues root tip organization weakly even when expression is driven by the *RCN1* promoter, demonstrating a more stringent requirement for A subunit function in the root apical meristem. Although it is possible that high-level overexpression of PP2AA3 could suppress this stem cell defect, our data clearly indicate that RCN1-containing PP2A complexes effectively target the key substrates for root growth at physiological expression levels, while PP2AA3-containing complexes do not.

RCN1 may be the preferred interaction partner for C and B subunits most active in seedling roots. The Aa and Ab isoforms of mammalian PP2A differ in their binding activities (Zhou et al., 2003; Sablina et al., 2007). However, the positive regulatory effect of RCN1 also is consistent with the hypothesis that RCN1 plays a role in an activation cycle for Arabidopsis C subunits analogous to that of TPD3 in yeast, promoting interaction with PTPA/RRD (Hombauer et al., 2007). The modest effects of loss of PP2AA2 and PP2AA3 function could indicate that these scaffolds do not interact efficiently with PTPA/RRD-like activators, and therefore

do not have equivalent effects on overall PP2A activity, at least in the presence of functional RCN1.

**RCN1 plays an isoform-specific role in root development**

Our data provide new insight into the developmental effects of increased phosphorylation of RCN1-specific PP2A substrates, demonstrating that loss of RCN1 regulation alters stem cell function and auxin distribution without producing the meristem collapse phenotype caused by more drastic reductions in PP2A activity. Previous studies have documented ectopic expression of cell identity markers and abnormal cell division patterns caused by treatment with exogenous auxin or loss of auxin transporter function in shoots and roots (Sabatini et al., 1999); (Benkova et al., 2003; Blilou et al., 2005). Our results indicate that meristem function is also sensitive to subtle changes in auxin flux, as well as to those more profound ones. Moreover, inhibition of phosphatase function during the seedling phase alone is sufficient to perturb meristem function, showing that root tip patterning is a dynamic process that responds rapidly to altered phosphorylation levels.

*RCN1* plays a key role in maintaining the patterns of root meristem cell division and function. Decreased expression of the *DR5-GUS* reporter in *rcn1* roots and of the QC marker *AGL42-GFP* in cantharidin-treated roots suggests that normal establishment of the local auxin concentration maximum and full expression of QC identity require RCN1-regulated PP2A activity. Since positioning of a local

maximum in the auxin pool at the root apex is an important patterning determinant (Sabatini et al., 1999), increased basipetal auxin transport in *rcn1* could affect root tip organization by altering the position of the auxin concentration maximum or by more generally increasing flux through the auxin transport stream in the root tip. Consistent with this hypothesis, the strongly reduced expression of DR5rev-GFP that is observed in *rcn1 pp2aa3* and *rcn1 pp2aa2/+* root tips is rescued by NPA treatment (Fuglsang et al., 2006; Michniewicz et al., 2007).

Previous studies have revealed complex feedback loops connecting ethylene response with auxin homeostasis in roots (Stepanova et al., 2005; Chilley et al., 2006). Furthermore, increased ethylene response was recently shown to stimulate cell divisions in the QC (Ortega-Martinez et al., 2007). Two observations argue against the hypothesis that increased ethylene response accounts for aberrant QC function in *rcn1*. First, we observe altered root tip morphology in light-grown seedlings, while increased ethylene synthesis was observed only in dark-grown *rcn1* seedlings (Muday et al., 2006). Second, although *rcn1* may enhance ethylene sensitivity in shoots (Larsen and Chang, 2001), *ctr1* root phenotypes are suppressed in the *rcn1 ctr1* double mutant (Larsen and Chang, 2001); ADL, unpublished data) suggesting that loss of *rcn1* reduces ethylene response in roots. Interestingly, ethylene treatment stimulates auxin accumulation in wild-type root tips, and mutations that reduce ethylene-induced auxin production confer weak ethylene insensitivity (Stepanova et al.,

2005). Thus increased basipetal auxin transport also may result in decreased sensitivity to ethylene in *rcn1* roots.

**Subcellular localization of RCN1 and A3 proteins**

Our data suggest that nuclear localization of RCN1 is developmentally regulated in roots. YFP-RCN1 was abundant in perinuclear and cytoplasmic compartments, but was under-represented in nuclei of meristematic and central elongation zone cells (Figure 5). Nuclear localization was observed in more mature cells in and above the proximal elongation zone, with little or no perinuclear accumulation evident in these cells. These data are consistent with the idea that RCN1 localization is dynamic during root cell differentiation, with enrichment in the perinuclear compartment in rapidly dividing cells and nuclear enrichment in post-mitotic cells. Interestingly, a GFP fusion to the OXIDATIVE SIGNAL-INDUCIBLE (OXI1) protein kinase, a member of the AGC kinase family that also includes the PINOID kinase, suggests that subcellular localization of OXI1 in root hairs also may be developmentally regulated, with nuclear accumulation occurring late in root hair development (Anthony et al., 2004; Rentel et al., 2004). Like RCN1, OXI1 plays a role in oxidative stress response; OXI1 activity increases under oxidative stress conditions, and promotes pathogen resistance and root hair development. Developmentally regulated localization of kinase/phosphatase pairs would provide an additional level of fine-tuning in phosphorylation-based control circuits.

We also observed peripheral localization of YFP-RCN1 and YFP-PP2AA3 in all cell types in the root tip. Membrane association appeared fairly uniform around the cell periphery, and did not exhibit obvious polarity or asymmetry in these experiments. Our cell fractionation data are consistent with the existence of a significant pool of membrane-associated PP2A in seedlings. Membrane association of PP2A complexes has been reported in several contexts previously, including early mouse development, during tight junction formation, and during associations with endothelial nitric oxide synthase at the plasma membrane (Gotz et al., 2000; Nunbhakdi-Craig et al., 2002; Wei and Xia, 2006). Arabidopsis PP2A interacts with the C-terminus of the plasma membrane ATPase AHA2 in vitro and exhibits partial co-localization with the PIN1 and PIN2 proteins in roots (Fuglsang et al., 2006; Michniewicz et al., 2007). Additionally, RCN1 protein binds phosphatidic acid, a lipid signaling molecule that recruits target proteins to the plasma membrane and plays a significant role in abiotic stress response (Meijer and Munnik, 2003; Testerink et al., 2004). Recruitment of PP2A to a membrane compartment via phosphatidic acid binding could alter phosphatase activity towards membrane-associated substrates. Membrane-associated PP2A activity may be critical for regulation of auxin transport, stress response and regulation of stem cell function in roots.

**Stress sensitivity in *rcn1* seedlings**

The data presented here indicate that *RCN1* function plays a unique role in mediating root stress response. Our working model states that positive

regulation of PP2A activity by the RCN1 protein contributes to a response that maintains normal growth under a wide range of stress conditions. As a modulator of auxin, ethylene and ABA levels and/or responses, *RCN1* is well positioned to act as an integrator of stress signaling. Abiotic stress may alter the cellular localization and amount of PP2A activity, resulting in PP2A-induced alterations in hormone responses. Loss of *RCN1* function compromises these adaptive alterations in PP2A localization and/or activity, leading to increased growth inhibition under stress conditions.

Previous studies focusing on two PP2A interactors, TAP46 and the AtCHIP E3 ubiquitin ligase, suggested that PP2A may play a role in the chilling response in Arabidopsis (Harris et al., 1999; Luo et al., 2006). Additionally, gene expression studies show that mRNAs for PP2A catalytic subunits in rice are differentially expressed in response to drought, salinity, and heat stress (Yu et al., 2003). An Arabidopsis PP2A catalytic subunit mutant was recently found to affect ABA-related stress responses through negative regulation of ABA signaling. While loss of *PP2AC-2* function confers ABA hypersensitivity (Pernas et al., 2007), loss of *RCN1* function results in reduced ABA sensitivity (Kwak et al., 2002). Paradoxically, both mutants show increased sensitivity to NaCl treatment. However, the *pp2ac-2* mutant exhibits a sensitivity phenotype specific for ABA-related stress (Pernas et al., 2007) unlike the general stress sensitivity phenotype reported here for *rcn1*. These disparities indicate that the effect of

*rcn1* loss of function is unlikely to be mediated by a specific effect on regulation of PP2AC-2 activity.

The parallel stress sensitivity of *rcn1* plants and *tpd3* yeast is striking, but it is not clear that the mechanism involved in the plant and yeast stress responses is similar. Interestingly, the transition between perinuclear enrichment and intra-nuclear YFP- RCN1 localization occurs in cells of the elongation zone, the same cell population that would be responsible for altered elongation under stress conditions. In yeast, PP2A is required for nuclear accumulation of the stress-responsive transcription factor Msn2p after nutrient deprivation, rapamycin treatment and temperature and ionic stress (Santhanam et al., 2004). Regulation by PP2A has been proposed to involve dephosphorylation of a nuclear export signal in Msn2p. Although we cannot rule out a similar explanation for the stress sensitivity of *rcn1* seedlings, there are no obvious orthologs of the Msn2 and Msn4 transcription factors in the Arabidopsis genome (ADL, unpublished). The Arabidopsis C2H2 zinc finger proteins that produce the best BLAST scores against Msn2 and Msn4 are more closely related to the TFIIIA family of transcription factors than to Msn2p or Msn4p. Additionally, we assayed for but did not observe an enhancement of nuclear RCN1 localization under stress conditions (see Supplemental Figure S7). Over a range of salt treatment times from minutes to 18 hours we did not detect any alteration in the nuclear vs. perinuclear YFP- RCN1 localization pattern, though some experiments suggested enrichment of the membrane-associated population after short-term

salt treatment (J.J.B. and A.D.L. unpublished). Additional biochemical experiments will be required to explore this possible membrane recruitment more rigorously.

## MATERIALS AND METHODS

### Plant material and growth conditions

The *rcn1-1* allele (Garbers et al., 1996) and derived transgenic lines (see below) were compared to the parental Ws line. An *rcn1-1* line homozygous for the *DR5-GUS* reporter (Rashotte et al., 2001) was backcrossed twice to the parental *DR5-GUS* line (Col background) or to *rcn1-1* and Ws to introgress the *rcn1-1* mutation or the reporter, respectively, into a more uniform genetic background. Plants homozygous for the *rcn1-1* mutation were identified among the self-progeny of the *DR5-GUS* back-cross products; homozygosity of the *DR5-GUS* marker was confirmed in self-progeny of these *rcn1* individuals. A similar procedure was used to isolate families homozygous for the DR5 reporter from the *rcn1-1* backcross. The *rcn1-6* allele is the T-DNA insertion allele SALK_059903 (kind gift of X. Wang and J. Chory) and is compared to the parental Col-0 line. *AGL42-GFP* (Nawy et al., 2005) was the kind gift of B. Kelley and P. Benfey.

Plants were grown as described previously (Zhou et al., 2004). For stress sensitivity measurements, seedlings were grown on vertical plates for four days in constant light at 24°C on standard medium (0.5 X MS salts containing 1% sucrose and 1% agar), then transferred to the same medium supplemented with the indicated salt, mannitol and hydrogen peroxide concentrations. Root tips of five mutant and five wild-type seedlings were aligned at a marked position on each plate and plates were returned to constant light. New growth was

measured seven days later using NIH ImageJ software, and relative new root growth was calculated as a percentage of that obtained on fresh standard medium. Each data point represents the average for fifteen seedlings. Hypocotyl elongation assays were performed as described previously (Deruère et al., 1999; Zhou et al., 2004). After scanning each plate to allow measurement of hypocotyl lengths, each seedling was scored for YFP fluorescence using a Leica MZFLIII dissecting microscope equipped with a mercury arc lamp and a GFP filter set.

**Construction of YFP fusions**

The *$ADC1_{pro}$:RCN1-YFP* fusions for yeast were constructed using the triple-template PCR strategy (Tian et al., 2004), with the *RCN1* cDNA (Garbers et al., 1996), the *ADC1* alcohol dehydrogenase promoter of pAAH5 (Ammerer, 1983) and pYFP3 (kind gift of D. Jackson) as templates for the partial PCR products. PCR primer sequences are given in Supplemental Table 1. The *$ADC1_{pro}$:RCN1-YFP* fusions were obtained and cloned into YEplac195 (Pitluk et al., 1995) using the Gateway and TOPO-XL systems (Invitrogen). *SUP35:GFP* was the kind gift of T. Serio (Brown University). The genomic *RCN1-YFP* and *YFP-RCN1* fusions for plant transformation also were constructed via TT-PCR using pYFP3 and wild-type Col-0 genomic DNA. To simplify cloning, the original TT-PCR products contained a short promoter region (861 bp total upstream from the *RCN1* ATG). The resulting fusion was cloned into pPZP221 (Hajdukiewicz et al., 1994) using the Gateway system (Invitrogen). The insert was sequenced and coding errors

were corrected by QuikChange II XL site-directed mutagenesis (Stratagene). A longer promoter fragment containing 2.1 kb of genomic sequence upstream from the start of the RCN1 transcript was amplified from Col-0 genomic DNA and substituted for the short promoter in the binary vector, yielding *RCN$_{pro}$:YFP-RCN* (*RYR*). To generate the *RCN$_{pro}$:YFP-PP2AA3* (*RYA*) fusion, the *PP2AA3* coding sequence was amplified from RAFL09-82-A21 (RIKEN BRC) and substituted for the *RCN1* coding region in *RYR*. For *PP2AA3$_{pro}$:YFP-PP2AA3* (*AYA*), a 1.2 kb *PP2AA3* promoter fragment was amplified from Col-0 genomic DNA and substituted for the *RCN1* promoter region in *RCN$_{pro}$:YFP-PP2AA3*. For *PP2AA3$_{pro}$:PP2AA3* the *YFP* coding sequence was deleted by oligonucleotide-mediated mutagenesis. All constructs were sequence verified and coding sequence errors were corrected by oligonucleotide -mediated mutagenesis. All clones were electroporated into *A. tumefaciens* strain GV3101 for transformation into *rcn1-1* plants via floral dip (Bechtold et al., 1993; Garbers et al., 1996). All transformants were selected for gentamycin resistance. T-DNA copy numbers were determined by screening gentamycin resistance segregation ratios followed by Southern blot analysis using a probe for the pPZP221 gentamycin resistance gene. The RYR-80, RYR-85, RYR-86, RRY-90, RRY-92, RYA-28, RYA-33 and AYA-2 lines carry single-copy T-DNAs, while the RYR-66, RYR-74, RYA-32, and AYA-6 lines carry two T-DNA copies and AYA-16 and AYA-100 carry three or more copies. The R2Q3 line carries three or more copies of the *RCN1-YFP* fusion driven by the short *RCN1* promoter fragment described above.

**Microscopy and detection of reporter gene expression**

For confocal imaging, seedlings were grown in constant light at 20°C on standard medium, stained lightly with propidium iodide (10μg $ml^{-1}$) or FM4-64 (5μg $ml^{-1}$) and mounted immediately for confocal analysis. For seedlings grown on NaCl, osmotic and ionic concentrations were maintained throughout the staining and mounting process. YFP fusion protein localization and seedling root tip morphology were examined by confocal microscopy using a Leica TCS SP2 AOBS spectral confocal microscope. To image YFP fusion proteins and root tip architecture, YFP fluorescence was excited at 514 nm and collected at 525 – 560 nm and propidium iodide fluorescence was excited at 593 nm and collected at 610 - 680 nm using a pinhole of 1 AU. To image *AGL42-GFP*, GFP fluorescence was excited at 488 nm and collected at 495 – 550 nm and FM4-64 fluorescence was excited at 593 nm and collected at 650 - 800 nm with a pinhole setting of 2AU and using sequential scanning. Images were processed using Leica Confocal Software (LCS Lite). To maintain comparable fluorescence signals, images were collected using constant beam intensities and settings within each experiment, unless otherwise noted. For starch staining, 4 d.p.g. seedlings were cleared and stained with I/KI (Fukaki et al., 1998) before imaging on a Zeiss Axiovert 200M. To detect *DR5-GUS* expression, seedlings were gently vacuum infiltrated and then stained overnight in 100 mM sodium phosphatase pH 7.0, 10 mM EDTA, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 0.1% Triton X-100, 1 mM X-glucuronide, followed by clearing in 90% ethanol. Yeast cells were imaged using a Zeiss Axioplan 2 equipped with a 100X

alphaPlan-FLUAR objective and Hamamatsu-ORCA camera (Hamamatsu Photonics), and images were collected with Openlabs software (Improvision).

**Yeast complementation assays**

Yeast strain Y1459 (*tpd3-1*; (van Zyl et al., 1992) was transformed with YEpTPD3 (kind gift of J. Broach), YEplac195, $ADH_{pro}$:RCN1, $ADH_{pro}$:YFP-RCN1, $ADH_{pro}$:RCN1-YFP and $ADH_{pro}$:$RCN1_{Y450H}$-YFP using a standard lithium acetate transformation protocol (Ausubel et al., 1992). For serial dilution assays, cultures were grown in YPD liquid medium at 30°C, diluted to an $OD_{600}$ of 2, then diluted in ten-fold steps into fresh YPD. Ten microliters of each dilution was spotted onto YPD medium or YPD supplemented with 600 mM NaCl. The SUP35:GFP strain (Satpute-Krishnan and Serio, 2005) was the kind gift of Tricia Serio.

**Preparation of Microsomal Membranes**

Microsomal membrane fractions were prepared as described (Blakeslee et al., 2007), with the following modifications. Dark-grown whole seedlings or roots were harvested at 5 d.p.g., ground, and microsomal membranes were isolated by spinning at 100,000 X g for 1 hr. Membrane fractions were washed twice, flash-frozen, and stored at -80ºC. For immunoblot analysis, membrane fractions were solubilized with 1% TritonX-100.

**Immunoblot analysis**

For immunoblot analysis, yeast cells were grown to log phase in YPD at 30°C, harvested by centrifugation, resuspended on ice in 100 mM Tris pH 7.5, 100 mM EDTA, 5 mM DTT, 2 mM PMSF, 5 mg/ml pepstatin, and 100 mg/ml aprotinin and leupeptin, and lysed by vortexing with glass beads. The resulting extracts were cleared by low-speed centrifugation and immediately boiled with Laemmli buffer. Plant extract preparation, SDS-PAGE and immunoblotting techniques were as described previously (Deruère et al., 1999), except that immunoblots were treated with 0.2 M NaOH at 37°C for 20 min immediately after transfer, followed by five PBST washes before blocking. Antisera used were anti-GFP (JL-8; Clontech), anti-PEP carboxylase (Rockland); anti-RCN1 (Deruère et al., 1999) and anti-C antibodies raised against the C-terminal peptide (CEPDTTRKTPDYFL) of Arabidopsis PP2A-C1.

**ACCESSION NUMBERS**

The Arabidopsis Genome Initiative locus identifiers for genes described in this article are as follows: *RCN1* (also known as *RegA*, *EER1*and *PP2AA1* ; At1g25490), *PP2AA3* (also known as *PDF2*; At1g13320), *PP2AA2* (also known as *PDF1*; At3g25800), *AGL42* (At5g62165).

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**FIGURES**

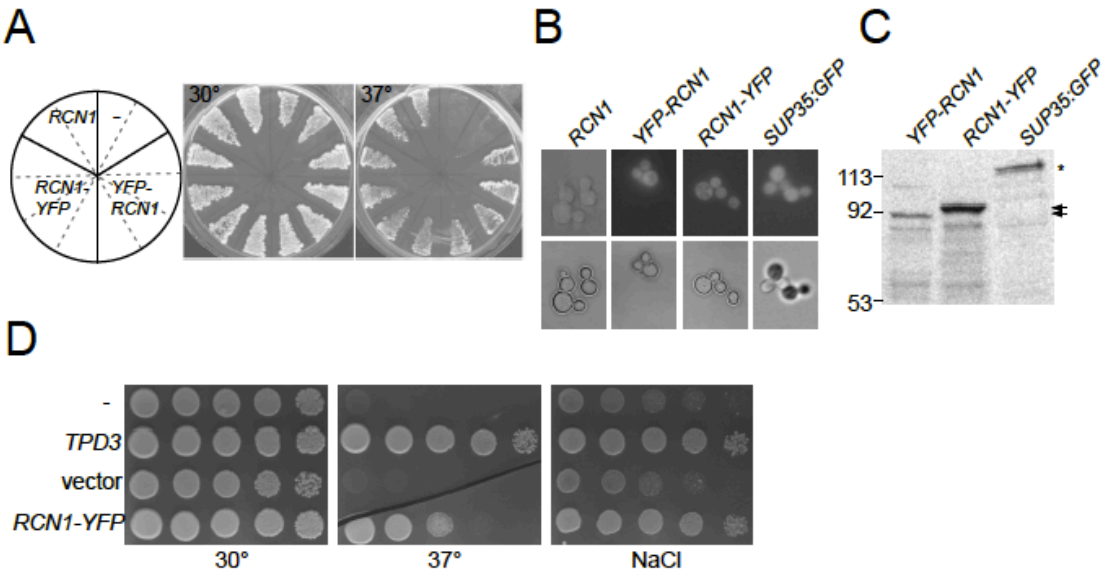


**Figure 1. *RCN-YFP* fusions supply regulatory A subunit function in yeast.** *RCN1-YFP* and *YFP-RCN1* fusions were expressed under control of the constitutive alcohol dehydrogenase promoter (Ammerer, 1983) in *tpd3-1* yeast cells (van Zyl et al., 1992). **(A)** Transformants carrying *RCN-YFP* fusions, an *RCN1* construct or the empty vector were streaked on duplicate YPD plates and incubated at 30° or 37° C. The diagram at left indicates the construct carried by cells in the corresponding sectors on both plates. **(B)** Cells carrying a native *RCN1* construct, *YFP-RCN1*, *RCN1-YFP* or a *SUP35:GFP* fusion (Satpute-Krishnan and Serio, 2005) were grown to early log phase and mounted for DIC (lower panel) or fluorescence (upper panel) microscopy. **(C)** Protein extracts of cells carrying the constructs indicated were subjected to SDS-PAGE and immunoblotting, using anti-GFP antibodies to detect the fusion proteins. The positions of the RCN1-YFP (solid arrows) and SUP35:GFP (asterisk) proteins are indicated at right. **(D)** Cells carrying the constructs indicated were grown in liquid culture and ten-fold serial dilutions were spotted on plates containing YPD medium or YPD plus 600 mM NaCl. YPD plates were incubated at 30°C or 37°C and YPD NaCl plates were incubated at 30°C.

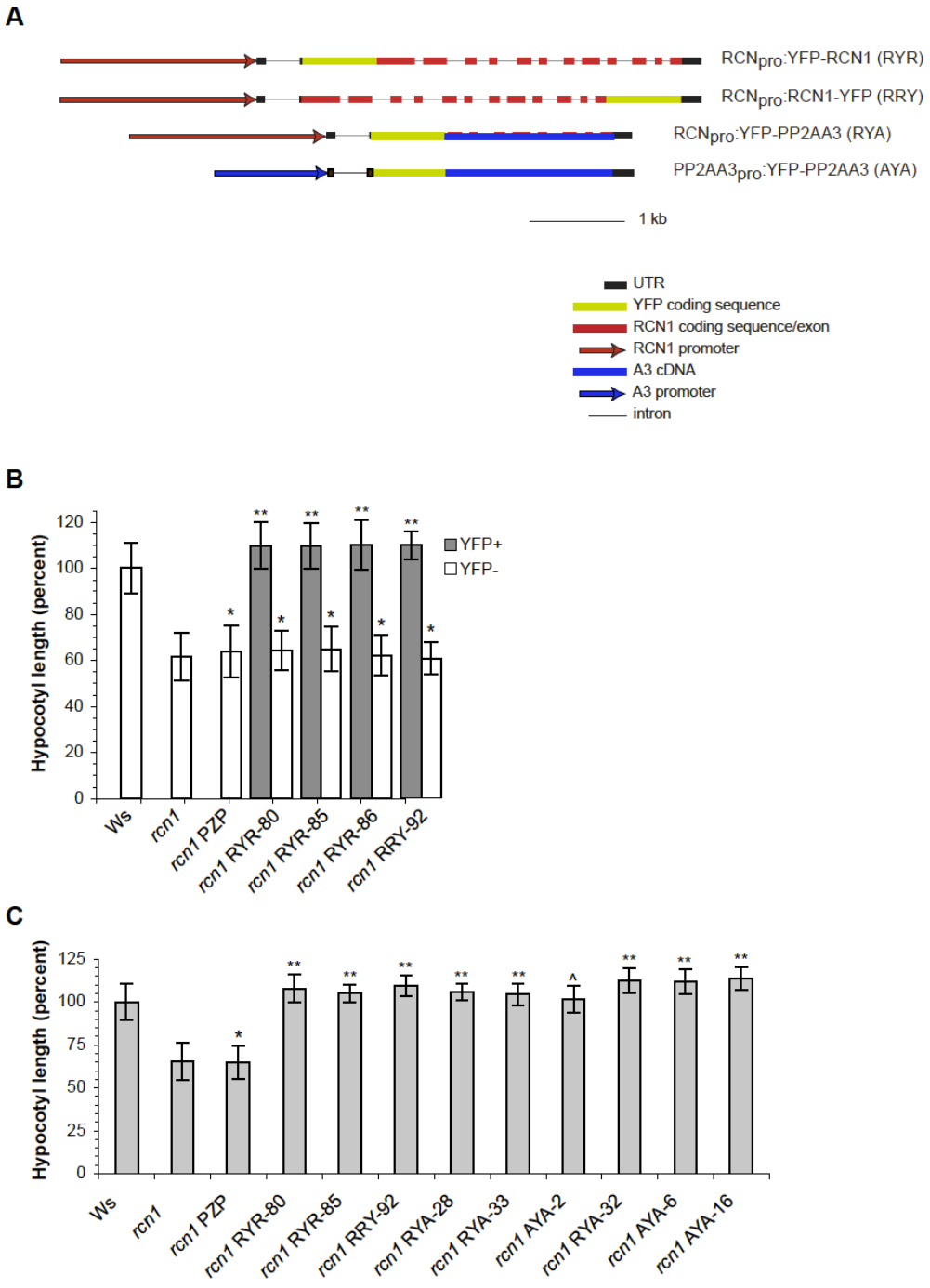


**Figure 2. *RCN1* and *PP2AA3* fusions to *YFP* rescue hypocotyl elongation in the *rcn1* mutant**. (**A**) The *YFP* coding sequence was fused at the N- or C-terminal end of the *RCN1* gene using a template overlap PCR strategy (see Materials and Methods) to generate in-frame fusions within the genomic *RCN1* sequence. In the *RCN$_{pro}$:YFP-PP2AA3* (*RYA*) construct, the *RCN1* coding sequence was replaced with the *PP2AA3* cDNA. The *PP2AA3$_{pro}$:YFP-PP2AA3* (*AYA*) construct was derived from *RYA* by substituting upstream sequences

from *PP2AA3* for the *RCN1* regulatory region. All constructs were transformed into the *rcn1-1* mutant. (**B**) Transformant families segregating for a single *YFP-RCN1* or *RCN1-YFP* transgene were scored for hypocotyl elongation and for YFP fluorescence after five days growth in the dark. Values shown represent the average hypocotyl lengths for each class ($YFP^+$ or $YFP^-$) relative to the wild-type control (Ws). Error bars indicate standard deviations; n > 25 for all controls; n > 45 for all segregating families. (**C**) Homozygous transformant families carrying the transgene constructs indicated were scored for hypocotyl elongation after five days growth in the dark. Values shown represent the average hypocotyl lengths for each family relative to the wild-type control (Ws). Error bars indicate standard deviations; n > 25 for all controls; n > 45 for all families carrying *YFP* fusion constructs. *rcn1* PZP is an *rcn1* transgenic line carrying the empty vector. Lines *rcn1* RYA-32 and *rcn1* AYA-6 each carry two copies of the fusion T-DNA, while line *rcn1* AYA16 carries three or more copies. All other lines carry the transgene construct in single copy. Levels of statistical significance as determined by Student's t-test: *, $p > 0.15$ vs. *rcn1* and $p < 10^{-6}$ vs. Ws; ** $p < 10^{-30}$ vs. *rcn1* and $p < 0.05$ vs. Ws; ^, $p < 10^{-30}$ vs. *rcn1* and $p > 0.8$ vs. Ws.

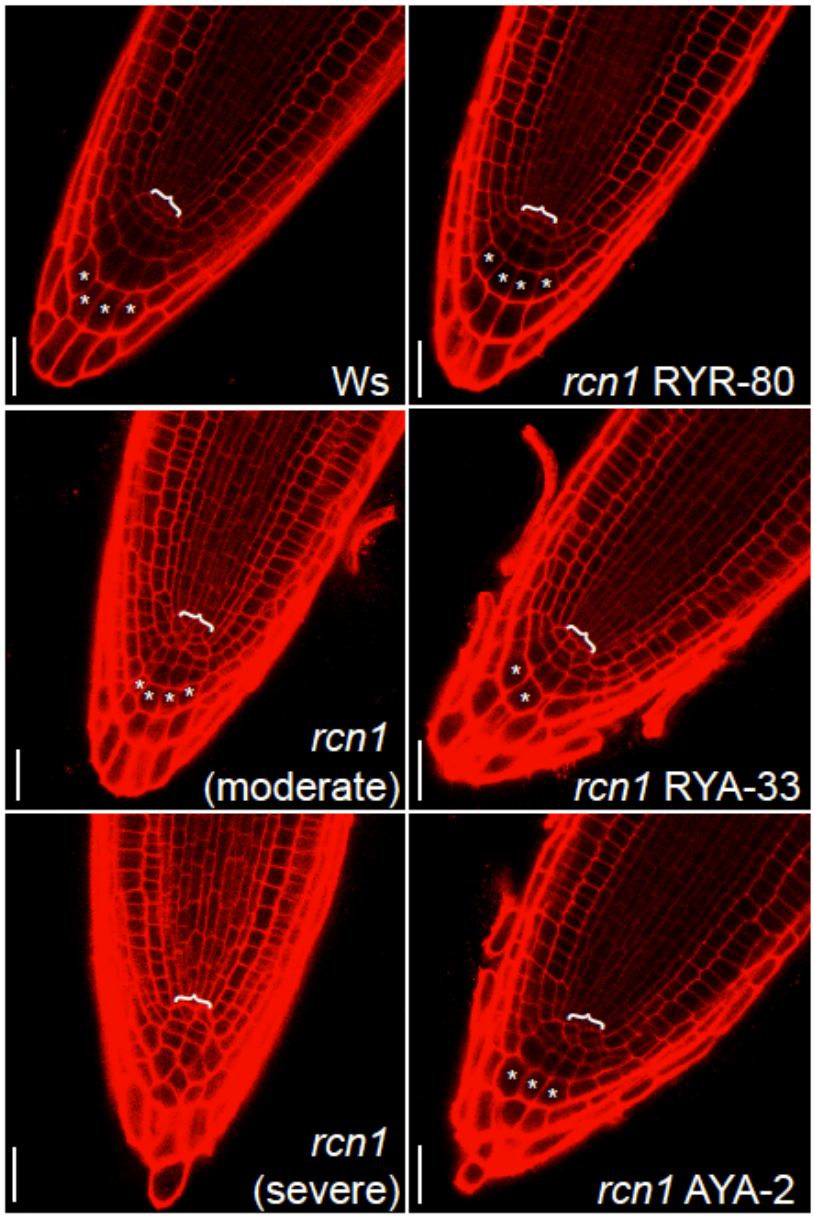


**Figure 3. Isoform specificity of *RCN1* function in root tip organization.** Median longitudinal sections of propidium iodide-stained 4 d.p.g. root tips were captured using confocal laser microscopy, revealing normal meristem organization in wild-type (Ws) roots and highlighting the disorganization of columella and initial cells in *rcn1* root tips. Examples of moderate (*rcn1* moderate) and severe (*rcn1* severe) *rcn1* disorganization phenotypes are shown. The *YFP-RCN1* fusion restores wild-type morphology in *rcn1* plants carrying the *RYR* construct, while the *YFP-PP2AA3* fusions carried in *RYA* and *AYA* transformants fail to fully rescue the *rcn1* phenotype. Brackets indicate the region of the QC; asterisks indicate cells representative of clearly defined columellar cell files. Scale bars, 25 mm.

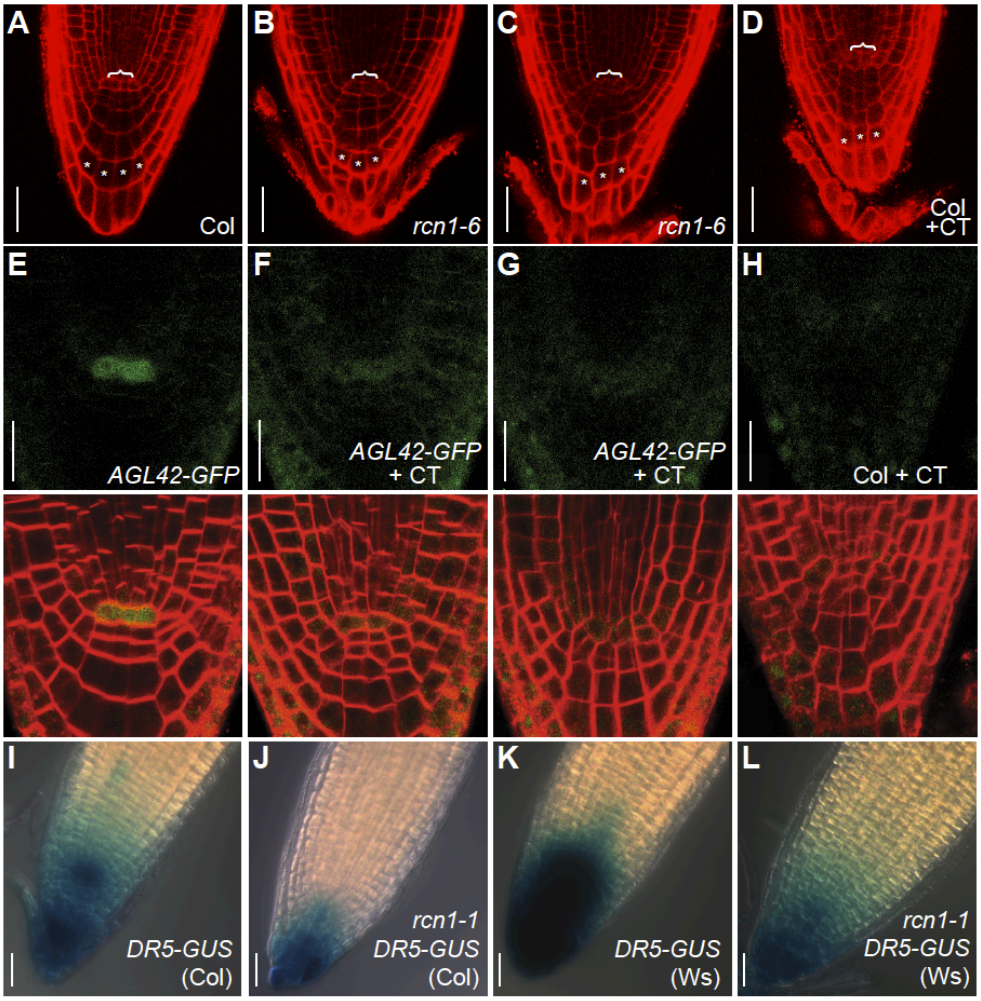


**Figure 4. Post-embryonic PP2A function maintains normal root tip development.** Median longitudinal sections of propidium iodide-stained 4 d.p.g. root tips were captured using confocal microscopy (A – D). One wild-type (A) and two representative *rcn1-6* mutant (B, C) root tips grown in the absence of cantharidin plus one wild-type root tip grown in the presence of 10 mM cantharidin (D) are shown. Cantharidin-treated wild-type roots show abnormalities around the QC (brackets) and reduced columellar cell file numbers matching those of *rcn1-6* mutant roots. Asterisks indicate cells representative of clearly defined columellar cell files. The *AGL42-GFP* reporter is expressed in QC cells in 4 d.p.g. seedling roots (E), but its expression is severely reduced in seedlings grown in the presence of 10 mM cantharidin (F, G). Each FM4-64-stained seedling was scanned sequentially for GFP fluorescence (upper row) and FM4-64 fluorescence (overlay shown in lower row). Under the imaging conditions used, a low level of background fluorescence is detected in wild-type Col root tips grown in the presence of cantharidin (H). *DR5-GUS* reporter activity (I – L) is reduced in roots of seedlings carrying the *rcn1-1* mutation in both the Col (I vs. J) and Ws (K vs. L) genetic backgrounds (see Materials and Methods). Scale bars, 25 mm (A-D, I - L) and 20 mm (E - H).

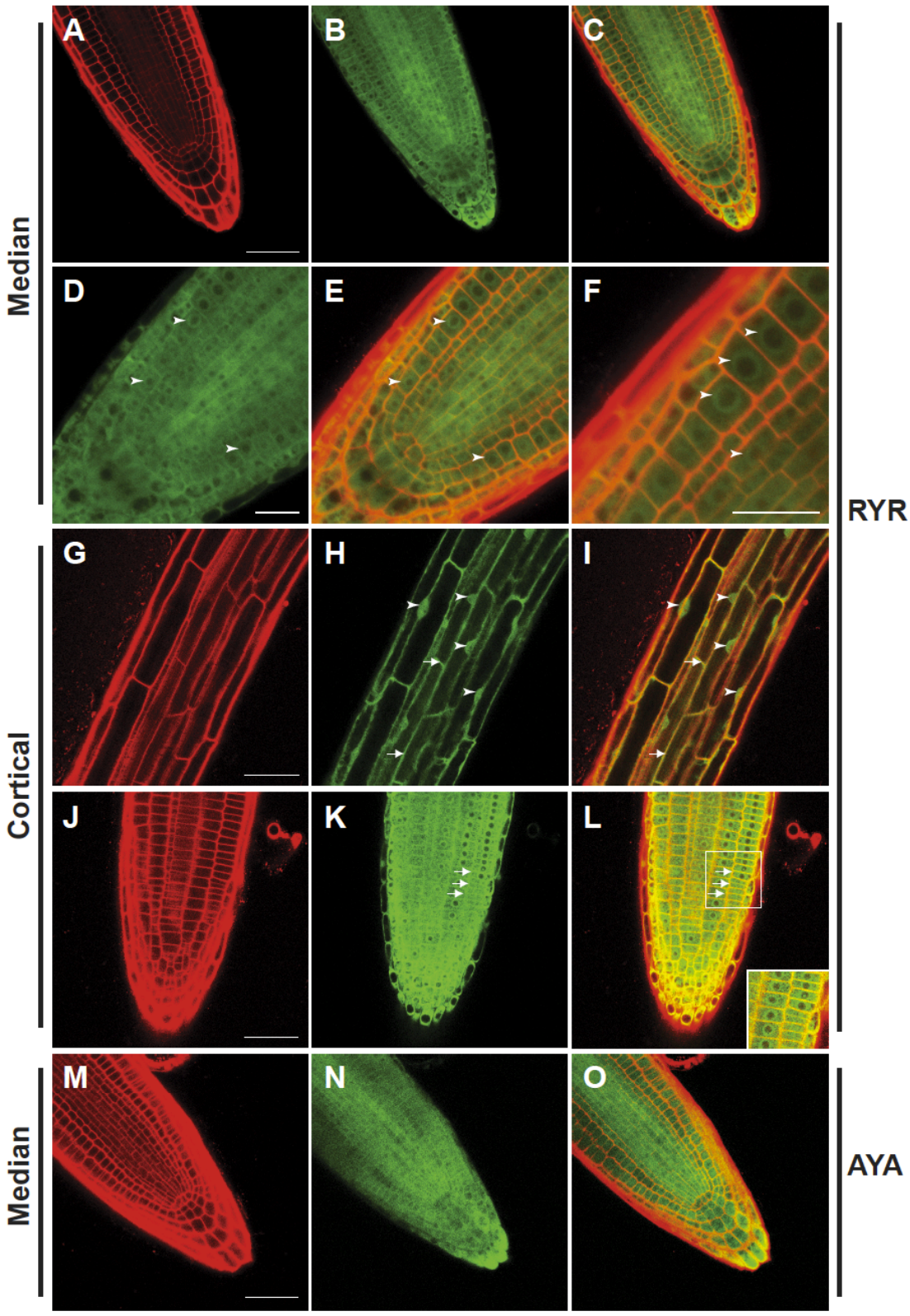
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
Median
Cortical
Median
RYR
AYA

**Figure 5. Abundance and localization of YFP-RCN1 protein in seedling roots.** Confocal microscopy reveals that the YFP-RCN1 fusion protein is abundant in all cell layers of the root tip (A-C) and shows cytoplasmic and perinuclear (arrowheads) localization (D – F) in cells of the apical meristem. In mature cortical cells (G – I), nuclear localization (arrowheads) is evident. Membrane association (arrows) is observed in both mature (G - I) and apical (J – L) cortical cells. The YFP-PP2AA3 fusion also exhibits ubiquitous accumulation in the root tip (M – O). Propidium iodide fluorescence (A, G, J, M) and YFP fluorescence (B, D, H, K, N) are overlaid (C, E, F, I, L, O) in medial (A – F, M – O) and cortical (G – L) optical sections of 4 d.p.g. roots of lines *rcn1* RYR-80 (A – L) and *rcn1* AYA-2 (M – O). Beam intensity was increased for imaging YFP fluorescence in line *rcn1* AYA-2 (N – O; compare Supplemental Figure S4E - F). Scale bars, 25 mm (A – C, G – O) and 10 mm (D – F).

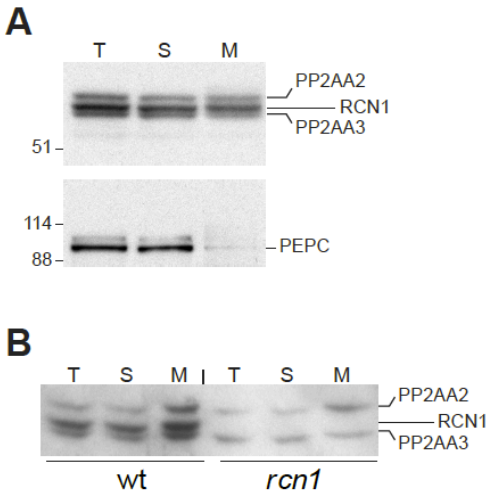


**Figure 6. Subcellular fractionation of native A subunit isoforms.** Soluble and microsomal membrane fractions were prepared from whole wild-type seedlings (**A**) or roots of wild-type and *rcn1* plants (**B**), and subjected to SDS-PAGE and immunoblotting analysis using anti-RCN1 (upper panel) and anti-PEPC (lower panel) antibodies. T, total; S, soluble; M microsomal membrane.

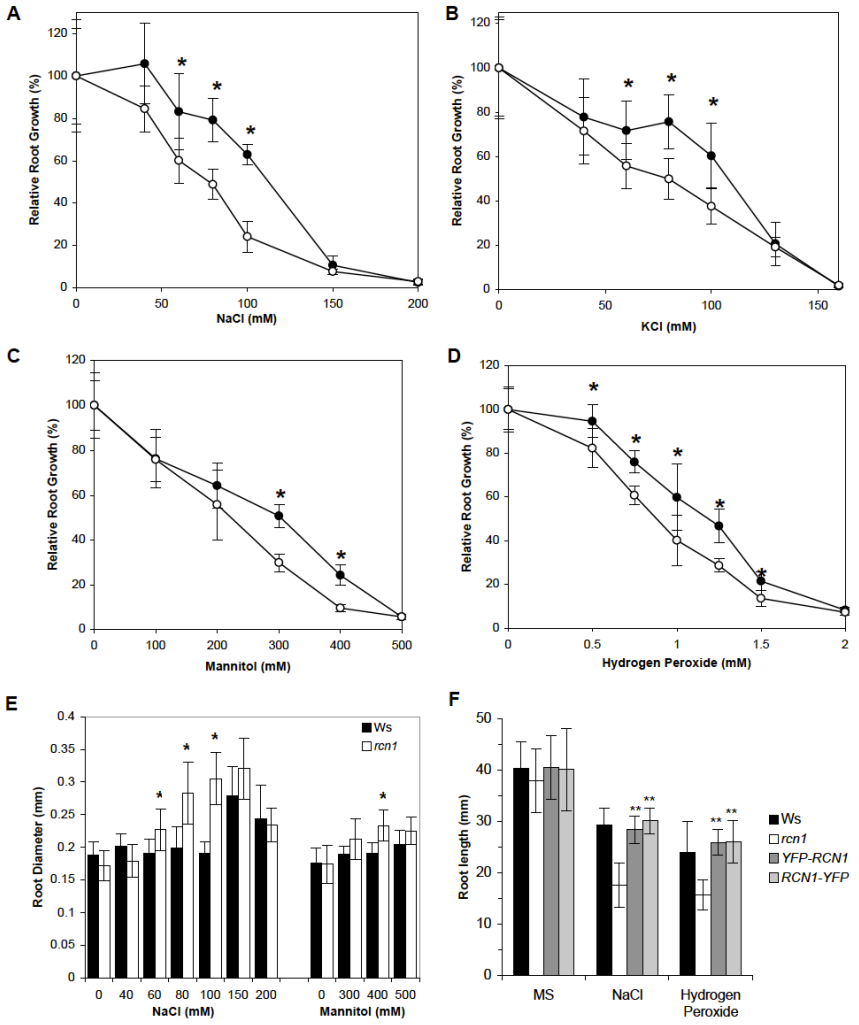


**Figure 7. Stress sensitivity is increased in *rcn1* seedlings.** Wild-type (closed symbols) and *rcn1-1* mutant seedlings (open symbols) were transferred from standard MS medium to plates containing the indicated concentrations of NaCl **(A),** KCl **(B)**, mannitol **(C)** and hydrogen peroxide **(D)**. New growth (elongation from the point of transfer) was measured after seven days additional growth. **(E)** Root diameter at the midpoint of the new growth segment was measured for plants grown on NaCl and mannitol. (**F**) Overall root length was measured on wild-type, mutant and complemented mutant seedlings (transgenic lines R1H9 and R2Q3; see Materials and Methods) transferred to NaCl- or hydrogen peroxide-containing plates as described above. For all panels, each value shown represents the average for twelve to fifteen seedlings; error bars indicate standard deviations. Asterisks indicate highly significant differences ($p < 0.002$ by Student's t-test). Asterisks indicate levels of statistical significance as determined by Student's t-test: * $p < 0.002$ for *rcn1* vs. wild-type; ** $p < 10^{-7}$ vs. *rcn1* and $p > 0.2$ vs. Ws.

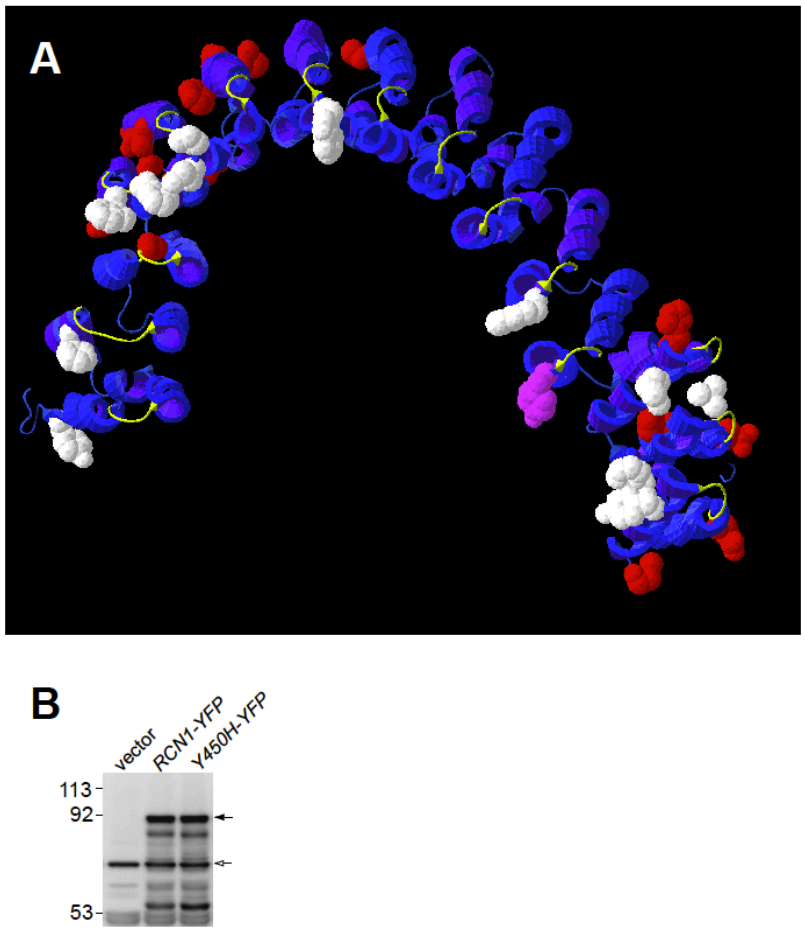


**Figure S1. Structural model of sequence differences between Arabidopsis A subunit isoforms.** (A) A structural model of the RCN1 protein was generated by threading the RCN1 (NP_173920) amino acid sequence into the structure of the human Aα regulatory subunit protein (Groves et al., 1999; PDB 1B3U). Alpha-helical regions of each HEAT repeat are shown in royal blue, with intra-repeat loops highlighted in yellow-green. Hydrophobic residues contributing to the interaction interface (Ruediger et al., 1994; Groves et al., 1999) are with white space-filling side-chains. Positions at which non-conservative substitutions occur in the PP2AA2 (NP_189208) and PP2AA3 (NP_172790 sequences are shown with red space-filling side-chains, and Tyr450 is shown in fuchsia. The amino-terminus is on the left. (B) Protein extracts of yeast cells carrying the constructs indicated were subjected to SDS-PAGE and Immunoblotting as described in Figure 1, using anti-RCN1 antibodies to detect the fusion proteins. The positions of the RCN1-YFP (solid arrows) and Tpd3 (hollow arrow) proteins are indicated at right.

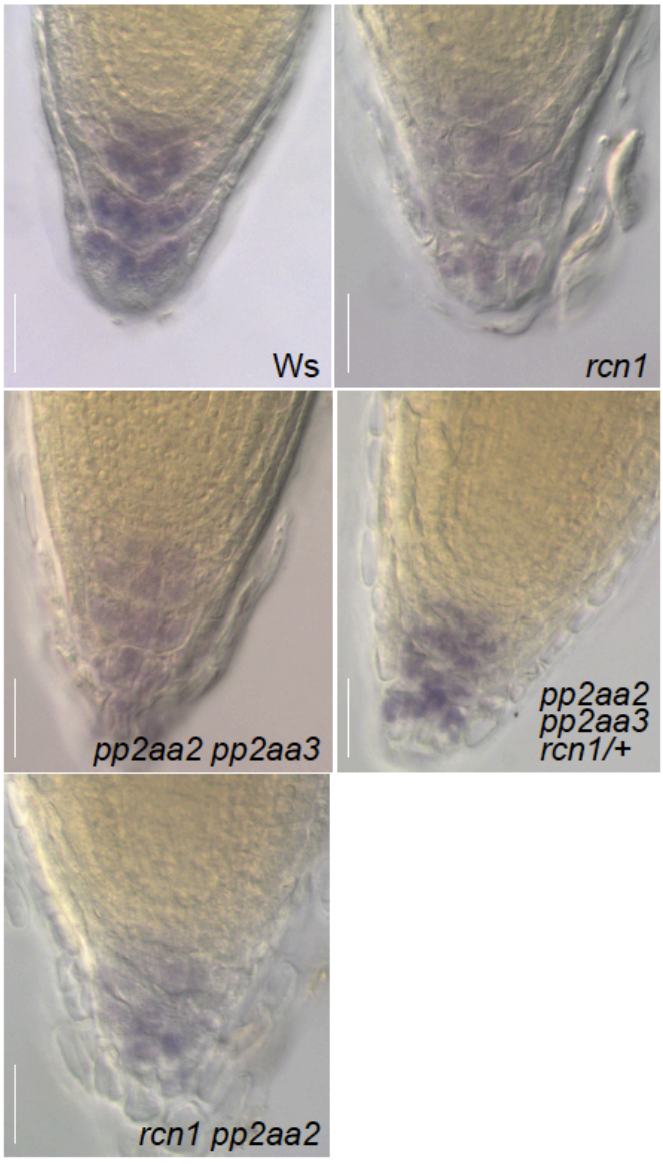


**Figure S2. *RCN1* is haploinsufficient in the *pp2aa2 pp2aa3* background.** Starch staining reveals normal columella organization in wild-type (Ws) and *ppraa2 pp2aa3* double mutant root tips, and abnormal organization in plants with reduced *RCN1* dosage (*rcn1* single mutant, *pp2aa2 pp2aa3 rcn1/+* and *rcn1 pp2aa2*). Scale bars, 25 μm.

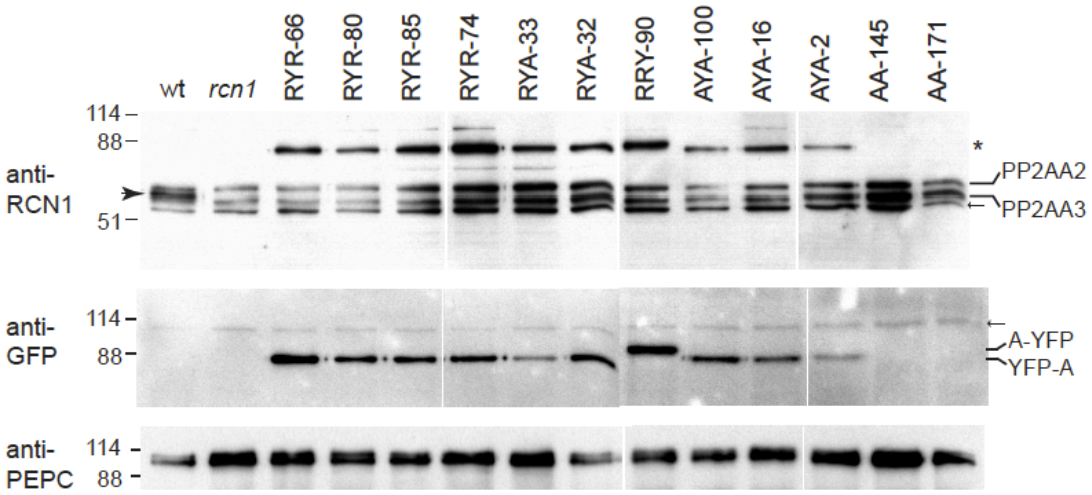


**Figure S3. Regulatory A subunit transgene products accumulate to native levels.** Protein extracts from roots of light-grown seedlings were subjected to SDS-PAGE and immunoblot analysis using anti-RCN1 (upper panel) and anti-GFP (middle panel) antibodies (see Materials and Methods). Because anti-RCN1 antibodies may not recognize RCN1 and PP2AA3 with equal affinity, the anti-GFP results provide a more accurate comparison of YFP-PP2AA3 and YFP-RCN1 abundance. Immunoblotting with anti-PEP carboxylase antibodies (PEPC; bottom panel) was used to control for protein loading. The endogenous RCN1 protein (arrowhead at left) migrates between the PP2AA2 and PP2AA3 proteins (indicated at right). Endogenous RCN1 protein is absent from extracts of all transgenic lines, since all constructs were transformed into the *rcn1-1* mutant. Asterisk, YFP-RCN1 and YFP-PP2A3 fusion proteins; arrows, non-specific bands.

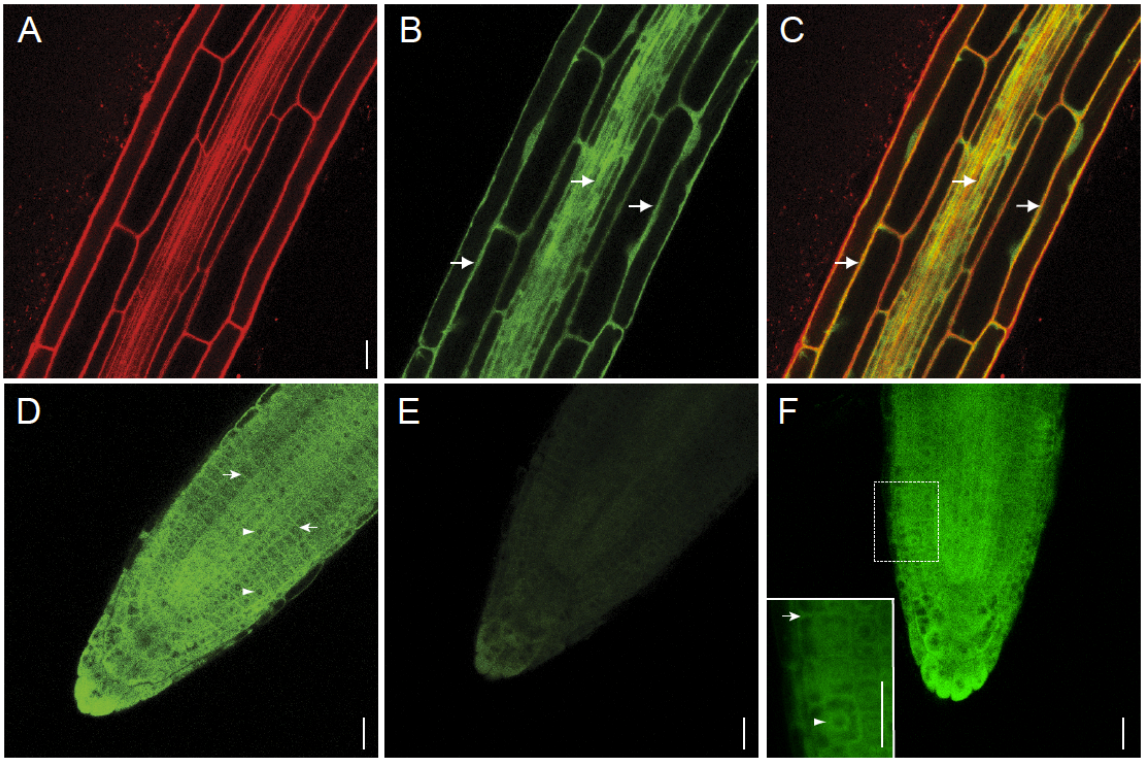


**Figure S4. Accumulation and localization of YFP-PP2AA3 fusion proteins.** YFP-RCN1 is abundant in vascular cells, and shows nuclear and peripheral (arrows) localization. Propidium iodide fluorescence (A) and YFP fluorescence (B) are overlaid (C) in a medial optical section of mature toot tissue from line *rcn1 RYR-80.* In root tips of the *rcn1 RYA-33* (D) and *rcn1 AYA* (E, F) lines, YFP-PP2AA3 fusion proteins shows similar distribution. Panels D and E were images with identical beam and exposure settings as used in Figure 5; panel F presents an *rcn1 AYA-2* root tip imaged at a higher laser beam intensity. Inset panel shows a close up of the boxed area. The YFP-PP2AA3 fusion shows perinuclear (arrowheads) and peripheral (arrows) localization in both lines. Scale bars, 20 μm.

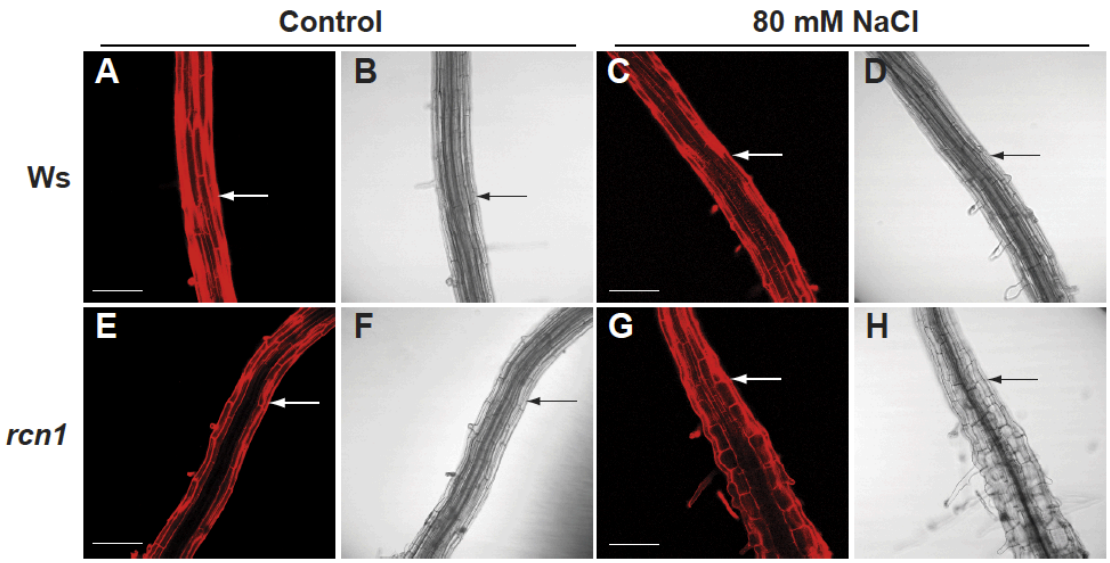


**Figure S5. Expansion of cortical cells in salt-stressed *rcn1* seedlings.** Wild-type (Ws) and *rcn1-1* seedlings were transferred to standard growth medium or medium containing 80 mM NaCl at 4 d.p.g. and were harvested and stained with propidium iodide for confocal imaging three days later. The position of the root tip at the time of transfer (white and black arrows) was marked on the Petri dish. Each pair of images shows a longitudinal section of the tissue flanking that site using confocal (A, C, E, G) or DIC (B, D, F, H) microscopy. The most mature cells are at the top of each panel; cells produced after the transfer to new medium are found below the white (or black) arrow in each panel. Scale bars, 150 μm.

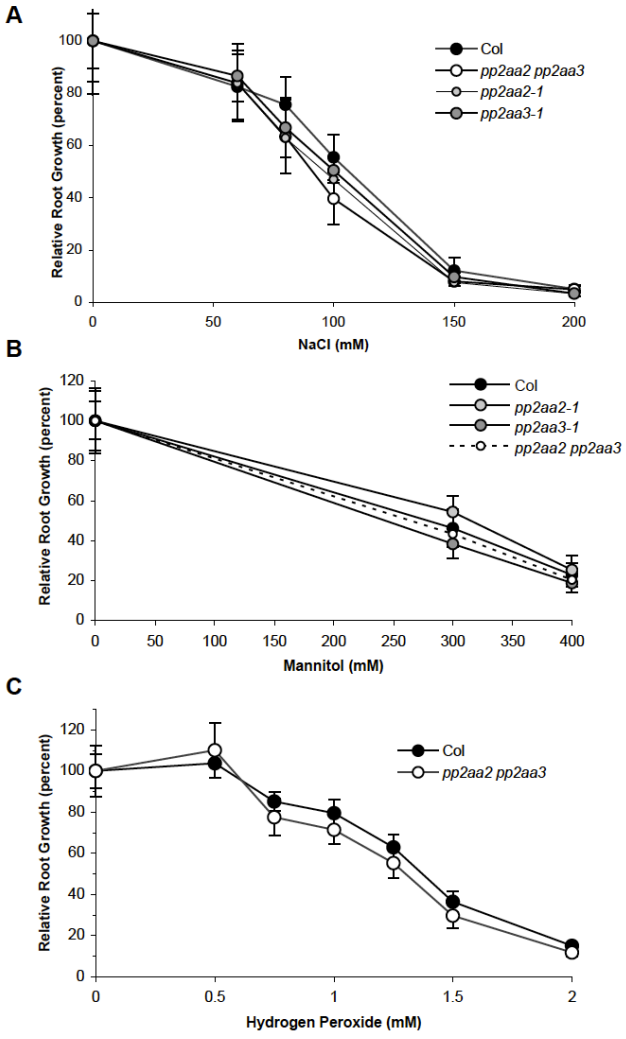


**Figure S6. Normal stress sensitivity in *pp2aa2* and *pp2aa3* mutants.** Wild-type (Col) and mutant (*pp2aa2-1, pp2aa3-1,* and/or *pp2aa2-1 pp2aa3-1*) plants were assayed for stress sensitivity as described in Figure 7.

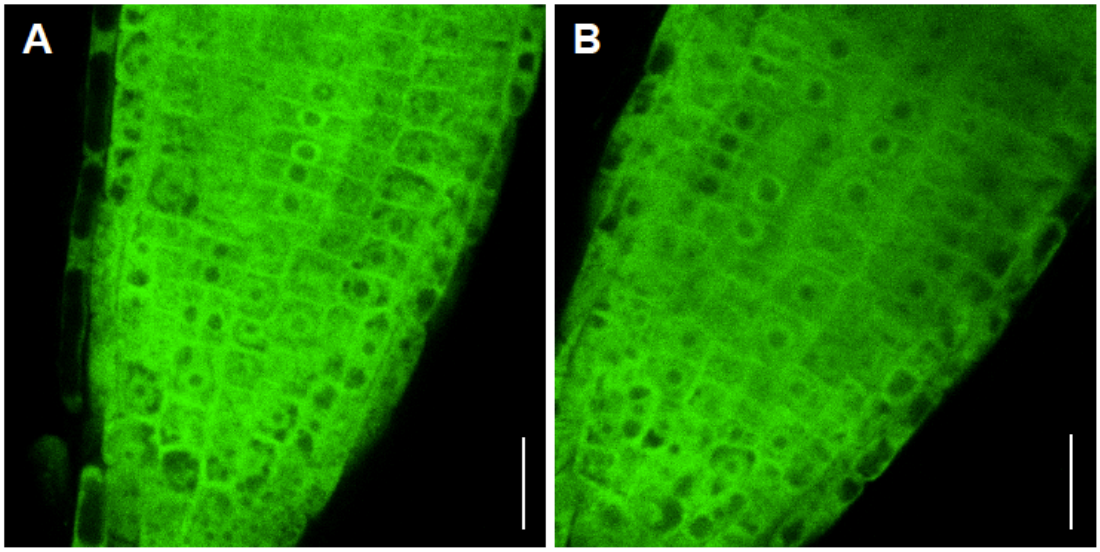


**Figure S7. Localization of a YFP-RCN1 fusion protein after NaCl treatment.** Subcellular localization of YFP-RCN1 in cortical cells of *rcn1 RYR-80* seedling roots was visualized using confocal microscopy after a 20-minute treatment with water (A) or 80 mM NaCl (B). As expected, water-treated control seedlings exyibited cytosolic, perinuclear, and peripheral membrane localization of the fusion protein. Increased nuclear localization of YFP-RCN1 was not detected in NaCl-treated seedlings. Scale bars, 20 μm.

## Table S1. PCR primers.

PCR Primers All primers are listed 5' to 3'.

| | |
|---|---|
| | **PLANT TTPCR PRIMERS (N- AND C-TERMINAL RCN1 FUSIONS)** |
| oP1g | CACCCGATTTCAAAGAACAAGTTAGCGAG |
| oP2g | TCCACCTCCACCTCCAGGCCGGCCCATAGCCATCTTATGTGAAATTCGA |
| oP3g | TGGTGCTGCTGCGGCCCTGGGGCCGCTATGGTAGATGAACCGTTGTATCC |
| oP4g | TGACGACCGCTTGATTAGCCT |
| oP2y | TCCACCTCCACCTCCAGGCCGGCCGGATTGTGCTGCTGTGGAACC |
| oP3y | TGGTGCTGCTGCGGCCGCTGGGGCCACAGCAGCACAATCCTGAATTGTT |
| | **PLANT TTPCR PRIMERS (YFP-PP2AA3 FUSION PRIMERS)** |
| oA3Not5 | GCTGCGGCCGCTGGGGCCTCTATGGTTGATGAGCCTTTATACC |
| oA23Cla3 | GCGATCGATTTAGCTAGACATCATCACATTGTCAATAG |
| | **YEAST TTPCR PRIMERS** |
| oP4ADH2 | ATACCTGAGAAAGCAACCTGACCTAC |
| oP1ADH3 | CACCGCCGGTTGGGGTTGCGATGATGAC |
| | **YFP PRIMERS** |
| oYFPleft | GGCCGGCCTGGAGGTGGAGGTGGAGCTGTGAGCA |
| oYFPright | GGCCCCAGCGGCCGCAGCAGCACCAGCAGGATC |
| | **ATTBP CLONING** |
| oP1BPG | GGGGACAAGTTTGTACAAAAAAGCAGGCTCGATTTCAAAGAACAAGTTAGCGAG |
| oP4BPG | GGGGACCACTTTGTACAAGAAAGCTGGGTTGACGACCGCTTGATTAGCCT |
| oP1BPY | GGGGACAAGTTTGTACAAAAAAGCAGGCTGCCGGTTGGGGTTGCGATGATGAC |
| oP4BPY | GGGGACCACTTTGTACAAGAAAGCTGGGTCCTGTGGATCCTCTAGATTGGCCGCCCTTT |
| | **RCN1 PROMOTER PRIMERS** |
| oSbfI | CATTATCAGTATTTTCCCTGCAGGTTGCACTATTTCTCTC |
| oRCN1.4 | GGGATACAACGGTTCATCTACCATAGC |
| oA3F | GCTGCGGCCGCTGGGGCCTCTATGGTTGATGAGCCTTTAT |
| oA23R | CAATTCAGGATTGTGCTGCTGTTTAGCTAGACATCATCACATTG |
| | **YFP DELETION PRIMER** |
| oYFPdel | GTTTTGGTGAGGATGTCTATGGTTGATGAGCCTTTATACCCG |
| | **Y450H MUTAGENESIS** |
| oT22C | CTGCAAGACAAGGTCCACTCTATCCGCGAAGC |
| oantiT22C | GCTTCGCGGATAGAGTGGACCTTGTCTTGCAG |
| oANSHT22C | GCTTCGCGGATAGAGTG |

# CHAPTER 3: PROTEIN PHOSPHATASE 2A CONTROLS ETHYLENE BIOSYNTHESIS BY DIFFERENTIALLY REGULATING THE TURNOVER OF ACC SYNTHASE ISOFORMS

**Kyle R. Skottke**, Gyeong Mee Yoon, Joseph J. Kieber and Alison DeLong

PLoS Genetics 2011 Apr;7(4):e1001370. Epub 2011 Apr 21

I designed and performed all the experiments in this chapter with the exception of Figures 1B, 1D, 4, and S1.

**ABSTRACT**

The gaseous hormone ethylene is one of the master regulators of development and physiology throughout the plant life cycle. Ethylene biosynthesis is stringently regulated to permit maintenance of low levels during most phases of vegetative growth but allow for rapid peaks of high production at developmental transitions and under stress conditions. In most tissues ethylene is a negative regulator of cell expansion, thus low basal levels of ethylene biosynthesis in dark-grown seedlings are critical for optimal cell expansion during early seedling development. The committed steps in ethylene biosynthesis are performed by the enzymes 1-aminocyclopropane 1-carboxylate synthase (ACS) and 1-aminocyclopropane 1-carboxylate oxidase (ACO). The abundance of different ACS enzymes is tightly regulated both by transcriptional control and by post-translational modifications and proteasome-mediated degradation. Here we show that specific ACS isozymes are targets for regulation by protein phosphatase 2A (PP2A) during *Arabidopsis thaliana* seedling growth, and that reduced PP2A function causes increased ACS activity in the *roots curl in 1-N-naphthylphthalamic acid 1* (*rcn1)* mutant. Genetic analysis reveals that ethylene overproduction in PP2A-deficient plants requires *ACS2* and *ACS6*, genes that encode ACS proteins known to be stabilized by phosphorylation, and proteolytic turnover of the ACS6 protein is retarded when PP2A activity is reduced. We find that PP2A and ACS6 proteins associate in seedlings, and that RCN1-containing PP2A complexes specifically dephosphorylate a C-terminal ACS6

phosphopeptide. These results suggest that PP2A-dependent destabilization requires RCN1-dependent dephosphorylation of the ACS6 C-terminus. Surprisingly, *rcn1* plants exhibit decreased accumulation of the ACS5 protein, suggesting that a regulatory phosphorylation event leads to ACS5 destabilization. Our data provide new insight into the circuitry that ensures dynamic control of ethylene synthesis during plant development, showing that PP2A mediates a finely tuned regulation of overall ethylene production by differentially affecting the stability of specific classes of ACS enzymes.

## AUTHOR SUMMARY

Like animals, plants produce a number of substances that regulate growth and coordinate developmental transitions and responses to environmental signals. Ethylene gas is one such regulator of the plant life cycle, playing important roles in fruit ripening, pathogen defenses and the regulation of cell expansion. Because overall plant form is determined largely by the degree and directionality of cell expansion, ethylene is a crucial regulator of morphology, and ethylene production must be maintained at low levels during phases of rapid cell expansion, such as early seedling growth. Recent work has identified molecular mechanisms that target ethylene biosynthetic enzymes for proteolytic degradation; this degradation plays a key role in controlling ethylene production. Here we exploit the molecular genetic resources available in the *Arabidopsis thaliana* system to identify a highly conserved protein complex that dephosphorylates target proteins as a new component of the mechanism that regulates degradation of ethylene-producing enzymes. Our findings show that protein phosphatase 2A plays a nuanced role in this regulatory circuit, with both positive and negative inputs into the stability of specific proteins that drive ethylene biosynthesis. This work enhances our understanding of the mechanisms that enforce adaptive levels of hormone production in plants.

## INTRODUCTION

Ethylene gas is a crucial regulator of numerous aspects of plant development and physiology, including germination, seedling growth and morphology, organ senescence and fruit ripening, as well as stress and defense responses (Abeles et al., 1992). The biosynthetic capacity for ethylene production is nearly ubiquitous throughout the plant body, but biosynthesis is generally maintained at low levels through regulatory circuitry that confers tight control while allowing rapid and dramatic increases under conditions such as wounding or fruit ripening. Ethylene biosynthesis levels change in response to endogenous developmental cues as well as light, temperature, pathogens and other exogenous signals (reviewed in references (Wang et al., 2002; Chae and Kieber, 2005; De Paepe and Van der Straeten, 2005)).

Ethylene is derived from methionine via a well-characterized biosynthetic pathway (reviewed in references (Yang and Hoffman, 1984; Kende, 1993; Zarembinski and Theologis, 1994; Wang et al., 2002)) in which the first committed step, conversion of *S*-adenosyl methionine to 1-aminocyclopropane 1-carboxylate (ACC), is performed by the enzyme ACC synthase (ACS; see Figure 1). The final step is the conversion of ACC to ethylene, $CO_2$ and cyanide by ACC oxidase (ACO). Under some conditions (particularly in fruit and flowers), ACO activity may be rate limiting, but ACC synthesis is generally the rate-limiting step for ethylene production during vegetative growth. In seedlings, increasing ACS

protein levels drive ethylene synthesis to high levels (Chae et al., 2003; Joo et al., 2008; Christians et al., 2009).

ACS isozymes are encoded by a gene family comprising three subclasses defined by the absence or presence of C-terminal phosphorylation motifs (Figure 1). Type 1 ACS isozymes carry target sites for mitogen-activated protein kinase (MAPK) phosphorylation; this MAPK motif lies immediately downstream from a calcium-dependent protein kinase (CDPK) phosphorylation site (Hernández Sebastià et al., 2004; Liu and Zhang, 2004; Kamiyoshihara et al., 2010). Type 2 isozymes carry only the CDPK target motif, and type 3 isozymes carry neither target site. Phosphorylation of the type 1 isozymes ACS2 and ACS6 by stress-responsive MAPKs (MPK3 and MPK6) results in increased ethylene synthesis through protein stabilization (Liu and Zhang, 2004; Joo et al., 2008; Han et al., 2010). Unphosphorylated type 1 isozymes are rapidly turned over via a 26S proteasome-dependent pathway, and the non-catalytic C-terminal region containing the CDPK and MAPK phosphorylation motifs is sufficient to confer instability on reporter protein fusions (Joo et al., 2008). CDPK-mediated phosphorylation of LeACS2, a tomato type 1 ACS, was recently shown to stabilize the enzyme, leading to increased ACS activity and ACC content in wounded tomato tissue (Tatsuki and Mori, 2001; Kamiyoshihara et al., 2010).

Type 2 proteins are recruited by ETHYLENE-OVERPRODUCING1 (ETO1) and the ETO1-like EOL1 and EOL2 proteins for ubiquitin-dependent proteolysis

(Chae et al., 2003; Wang et al., 2004; Yoshida et al., 2005; Christians et al., 2009). Ethylene overproduction in etiolated *eto1* seedlings is caused by decreased proteolytic turnover of type 2 ACS isozymes. The dominant *eto2* and *eto3* mutations, which alter C-terminal amino acid sequences of ACS5 and ACS9 required for recognition by ETO1 and EOL proteins, also cause ethylene overproduction in etiolated seedlings. Each of these mutations stabilizes the $ACS^{eto}$ protein product by preventing its interaction with ETO1 (Chae et al., 2003; Wang et al., 2004; Yoshida et al., 2006; Christians et al., 2009). The CDPK target motif of type 2 ACS proteins can be phosphorylated, but the role of phosphorylation in regulating ACS accumulation has not yet been established (Hernández Sebastià et al., 2004; Christians et al., 2009).

Although phosphorylation by CDPK and MAPK kinases has been linked to stabilization of ACS isozymes, 'partner' phosphatases acting on ACS isozymes have not been identified. The PPM-type protein phosphatase AP2C1 negatively regulates MPK6, and overexpression of AP2C1 compromises wound-induced ethylene production and disease resistance (Schweighofer et al., 2007). Okadaic acid and calyculin, inhibitors of protein phosphatase 1 (PP1) and PP2A, stabilize LeACS2 in tomato, and okadaic acid treatment causes the accumulation of LeACS2 phosphorylated at the CDPK site (Kamiyoshihara et al., 2010).

The heterotrimeric serine/threonine protein phosphatase PP2A was implicated in regulation of ethylene synthesis when the PP2A-deficient *rcn1* mutant was

shown to overproduce ethylene (Larsen and Chang, 2001). Because ethylene is a potent inhibitor of cell expansion in dark-grown seedlings, ethylene overproduction results in a characteristic short hypocotyl phenotype in *rcn1* plants (Larsen and Chang, 2001; Muday et al., 2006; Blakeslee et al., 2008). The *RCN1* gene encodes one of three regulatory/scaffolding A subunits of Arabidopsis PP2A, and the pleiotropic *rcn1* mutant phenotype results from a significantly reduced level of PP2A activity in *rcn1* plants (Garbers et al., 1996; Deruère et al., 1999). The experiments described here were designed to test the hypothesis that sustained C-terminal phosphorylation of ACS isozyme[s] due to decreased PP2A activity might account for ethylene overproduction in *rcn1* plants. Our results show that PP2A negatively regulates the activity and accumulation of type 1 ACS isozymes. PP2A directly interacts with the ACS6 protein, and PP2A complexes dephosphorylate a carboxy-terminal ACS6 phosphopeptide in vitro. Dephosphorylation requires the PP2A complexes that contain the RCN1 scaffolding subunit. Elicitor-mediated activation of MPK6, which up-regulates ethylene synthesis via type 1 ACS isozymes, has a reduced effect in the *rcn1* mutant background, consistent with the model that the baseline accumulation of phosphorylated type 1 ACS proteins is increased in *rcn1* plants. Genetic and molecular data also show that PP2A positively regulates the abundance of type 2 ACS proteins, revealing an unexpected role for PP2A in promoting accumulation of type 2 ACS isozymes. Our findings provide new insight into the finely tuned and phosphorylation-dependent regulation of ethylene synthesis.

## RESULTS

### PP2A Inhibition Increases ACS Activity

Ethylene biosynthesis is enhanced in dark-grown *rcn1* seedlings (Larsen and Chang, 2001; Muday et al., 2006). Because ACC synthesis is generally the rate-limiting step for ethylene production during vegetative growth, we asked whether ACS activity was increased in *rcn1* mutant seedlings. We found that *rcn1* mutants in both the Columbia (Col) and Wassilewskija (Ws) genetic backgrounds exhibited increased ACS enzymatic activities in dark-grown seedlings (Figure 2). In the Col genetic background, *rcn1-6* and *eto1* seedlings showed similar ACS activity levels. These data suggest that ethylene overproduction and reduced hypocotyl lengths in *rcn1* mutant seedlings result at least in part from increased ACS enzymatic activity.

### PP2A-mediated Regulation of Ethylene Production Requires Type 1 ACS Isozymes

The *eto1* mutation causes ethylene overproduction by stabilizing type 2 ACS isozymes (Chae et al., 2003; Wang et al., 2004; Yoshida et al., 2005; Christians et al., 2009). In both *rcn1* and *eto1* mutants, ethylene overproduction reduces hypocotyl elongation in dark-grown seedlings, causing a short hypocotyl phenotype. To determine whether PP2A-mediated regulation of ethylene synthesis requires the ETO1 protein, we assayed hypocotyl elongation in *rcn1*

*eto1* double mutant seedlings. As expected, hypocotyl lengths in both single mutants were approximately one-half of those exhibited by the wild-type parents (Figure 3A). Seedlings carrying both the *rcn1* and *eto1* mutations exhibited an extreme reduction in hypocotyl length, corresponding to 25% of the wild-type parent. Double mutant seedlings also exhibited a significant increase in ethylene production above the level observed in either single mutant parent ($p < 0.001$; Figure 3B). These data indicate that the *rcn1* and *eto1* defects in regulation of ethylene synthesis are additive, suggesting independent modes of action for PP2A and ETO1. Although the ethylene overproduction defect of *eto1* seedlings is more severe than that of *rcn1* seedlings, the hypocotyl lengths of the two mutants are similar in our assays (Figure 3B vs. 3A). However, both the site (or source tissue) and timing of ethylene overproduction will affect the overall length of hypocotyls, and the *rcn1* and *eto1* mutants may differ significantly in these characteristics.

To determine whether PP2A-mediated regulation of ethylene synthesis targets particular ACS isozymes, we asked whether *acs* loss-of-function mutations affect the short hypocotyl phenotype caused by PP2A inhibition. Wild-type seedlings treated with the phosphatase inhibitor cantharidin (CT) exhibit a characteristic inhibition of hypocotyl elongation similar to that observed in untreated *rcn1* seedlings (Deruère et al., 1999; Larsen and Cancel, 2003; Blakeslee et al., 2008). Hypocotyl elongation in the type 1 *acs* single mutants *acs2* and *acs6* and in the *acs2 acs6* double mutant showed decreased cantharidin response (Figure

3C). These data suggest that the effect of cantharidin on hypocotyl elongation is dependent on type 1 ACS isozymes. Similarly, ACS enzymatic activity in *acs2 acs6* double mutant seedlings was insensitive to cantharidin treatment, while normal seedlings treated with cantharidin exhibited increased ACS activity relative to untreated controls (data not shown). Interestingly, *acs5* and *acs9* loss-of-function mutants showed slightly increased cantharidin response (Figure 3C), indicating that the type 2 isozymes ACS5 and ACS9 are not required for cantharidin-mediated inhibition of hypocotyl elongation.

We also asked whether *acs* loss-of-function mutations affect ethylene overproduction in the presence of cantharidin. Wild-type seedlings treated with cantharidin exhibited a 76% increase in ethylene production (Figure 3D). While cantharidin-treated *acs6* seedlings exhibited a similar increase in ethylene synthesis, both *acs2* and *acs2 acs6* double mutant seedlings showed a reduced response to cantharidin treatment. Unlike the *acs2* and *acs6* mutants, *acs5* and *acs9* mutant seedlings exhibited a reduced baseline level of ethylene production. However, both type 2 *acs* mutants also showed a greater stimulation of ethylene synthesis in the presence of cantharidin (Figure 3D). Both the decreased basal ethylene synthesis and the increased response to cantharidin were more pronounced in the *acs5 acs9* double mutant, which exhibited a three-fold increase in ethylene production when grown in the presence of cantharidin (Figure S1). As in the hypocotyl elongation experiment described above, our data suggest that phosphatase inhibition acts through type 1 ACS isozymes to

increase ethylene synthesis, while type 2 isozymes are not required for this effect. In addition, the data suggest that PP2A inhibition may have opposing effects on the activities of type 1 vs. type 2 isozymes. This hypothesis is discussed in more detail below.

**Ethylene Synthesis in *rcn1* Plants is Not Stimulated by Elicitor Treatment**

Phosphorylation-mediated stabilization of type 1 ACS isozymes can be induced by treatment with the bacterial elicitor Flg22 (Liu and Zhang, 2004; Joo et al., 2008). Because both the *rcn1* mutation and cantharidin treatment decrease PP2A activity levels, we expect that PP2A substrates will be hyperphosphorylated both in *rcn1* plants and in cantharidin-treated wild-type plants. We reasoned that the stabilizing effect of Flg22 treatment might be lessened if steady-state ACS phosphorylation levels are increased in the *rcn1* mutant. We measured Flg22-induced ethylene synthesis in wild-type (Col) and *rcn1-6* mutant seedlings, and found that *rcn1-6* seedlings indeed showed a reduced response to Flg22 treatment (Figure 4). While ethylene production rose by more than 2.5-fold (265%) in wild-type Col seedlings, the elicitor-induced increase in *rcn1-6* mutant plants (25%) was not statistically significant. Analysis of variance (ANOVA) shows that the difference between Flg22-treated wild-type (Col) seedlings and *rcn1-6* seedlings (Flg22-treated or untreated) also was not statistically significant. Flg22-induced ethylene synthesis in *rcn1-6* plants was not rescued by a higher Flg22 dose; treatment with 200 nM Flg22 resulted in a 262% increase in ethylene production in the wild type, and less than a 30% increase in

*rcn1-6* plants (data not shown). The attenuated effect of Flg22 treatment in *rcn1-6* seedlings is consistent with the hypothesis that type 1 ACS isozymes are more phosphorylated and more stable when PP2A activity is reduced.

**PP2A Inhibition Stabilizes ACS6 Protein**

To directly assess the effect of PP2A inhibition on ACS6 protein turnover, we assayed the stability of an epitope-tagged ACS6 protein (Joo et al., 2008) in wild-type and *rcn1* mutant seedlings, and in wild-type plants grown in the presence of cantharidin. As expected, myc-ACS6 was rapidly turned over in dark-grown seedlings (Figure 5A). The *rcn1* mutation retarded the turnover of myc-ACS6, as did cantharidin treatment (Figure 5A). In contrast, the phosphomimic allele myc-ACS6$^{DDD}$, which exhibits enhanced and phosphorylation-independent stability (Liu and Zhang, 2004), was equally stable in wild-type seedlings grown in the absence or presence cantharidin (Figure 5B). Thus, the wild-type ACS6 protein is stabilized by phosphatase inhibition, while the phosphomimic ACS6$^{DDD}$ protein is immune to this effect.

Immunoblots probed with anti-myc antibody revealed two poorly resolved bands near the molecular weight predicted for the myc-ACS6 protein. Extracts from *rcn1* mutants and from cantharidin-treated wild-type plants showed enhanced accumulation of the upper band (Figure 5A, C and D). To determine whether the upper and lower bands corresponded to phosphorylated and unphosphorylated myc-ACS6, we isolated protein extracts from *myc-ACS6*-expressing wild-type

and *rcn1* plants and treated aliquots with alkaline phosphatase (Figure 5C) or with PP2A complexes immunoprecipitated from plants expressing an RCN1-YFP fusion protein (Figure 5D). Treatment with either CIP or PP2A resolved the upper and lower bands into a single species running at the position of the lower band (Figure 5C and D). These results are consistent with the hypothesis that both the *rcn1* lesion and phosphatase inhibition result in the accumulation of a more stable phosphorylated ACS6 species in vivo.

**ACS6 Protein Interacts with PP2A**

We asked whether the ACS6 protein interacts with PP2A complexes in planta. To address this question, we used a reciprocal co-immunoprecipitation approach. First we used anti-myc antibodies to immunoprecipitate ACS6 from protein extracts isolated from plants expressing myc-ACS6$^{DDD}$ (Figure 6A). We probed these immunoprecipitates for regulatory A subunits of PP2A using anti-RCN1 antibodies that recognize all three A subunit isoforms (Deruère et al., 1999; Zhou et al., 2004). Regulatory A subunits were detected in the pellet fraction even after a detergent wash, suggesting a stable interaction between ACS6$^{DDD}$ and PP2A. When anti-RCN1 antibodies were used for the reciprocal co-immunoprecipitation, immunoblotting revealed the presence of the myc-ACS6$^{DDD}$ protein in the immunoprecipitates, and the ACS6 signal was maintained through a stringent detergent wash (Figure 6B). A weak but reproducible ACS6 signal was observed in anti-RCN1 immunoprecipitates isolated from plants expressing wild-type myc-ACS6; this signal was reduced after a gentle washing treatment,

and was nearly undetectable after a stringent wash (Figure 6C). These co-immunoprecipitation data suggest that PP2A directly interacts with ACS6, and that interaction with the low-abundance wild-type isoform is unstable, while the interaction with the stabilized and more abundant ACS6$^{DDD}$ protein is more robust. While the ACS6$^{DDD}$ protein is an imperfect proxy for the phosphorylated wild-type ACS6 protein in this experiment, PP2A interaction may require motifs outside the MAPK phosphorylation site that are unaffected by the DDD substitution. For instance, in the case of PP2A-mediated dephosphorylation of the brassinosteroid-responsive transcription factor BRASSINAZOLE-RESISTANT1 (BZR1), a distinct binding motif is required for the PP2A interaction that leads to dephosphorylation of multiple sites; some or all of these sites lie outside the binding domain (Tang et al., 2011).

**PP2A Dephosphorylates an ACS6 Peptide Substrate In Vitro**

To determine whether PP2A acts directly on ACS6, we asked whether immunoprecipitated PP2A complexes could dephosphorylate an ACS6 phosphosubstrate. Because both native and recombinant ACS6 proteins are very unstable, we used a synthetic C-terminal peptide in our dephosphorylation assays. A C-terminal ACS6 30mer containing the three MPK target motifs was phosphorylated with recombinant MKK4$^{DD}$ and MPK6 (Liu and Zhang, 2004) and then used as a substrate for immunoprecipitated PP2A complexes isolated from transgenic plants expressing a YFP-tagged RCN1 (Figure 7A). PP2A immunocomplexes showed dephosphorylation activity against the ACS6 C-

terminal peptide (Figure 7B). When the phosphatase inhibitor okadaic acid was added to the dephosphorylation reactions, activity dropped to background levels (data not shown). In combination with our finding that ACS6 and PP2A interact physically (Figure 6), these data suggest that ACS6 is a bona fide PP2A substrate in etiolated seedlings.

To determine whether a specific population of PP2A complexes may dephosphorylate ACS6, we tested the activities of crude lysates extracted from plants carrying mutations in genes encoding the three scaffolding A subunits of PP2A. Plants carrying the *rcn1* mutation maintain expression of the PP2AA2 and PP2AA3 scaffolds, while plants carrying the *pp2aa2-1 pp2aa3-1* double mutant combination express only the RCN1 scaffold (Zhou et al., 2004). Okadaic acid-sensitive ACS6 dephosphorylating activity was observed in extracts from wild-type and *pp2aa2-1 pp2aa3-1* seedlings, but not in extracts from *rcn1* plants (Figure 7C). In replicate experiments, extracts from *pp2aa2-1 pp2aa3-1* mutants showed 78 ± 7% of wild-type activity levels, while *rcn1* mutants showed only background (0 ± 1%) levels of activity (n = 4). These data indicate that RCN1-containing PP2A complexes are required for dephosphorylation of the ACS6 C-terminus. In contrast to the results obtained with this ACS6 peptide substrate, *rcn1* mutant plants exhibit approximately a two-fold decrease in 'bulk' PP2A activity (measured against the model substrate myelin basic protein; (Deruère et al., 1999; Muday et al., 2006)), while *pp2aa2-1 pp2aa3-1* plants exhibit 89 ± 5% of wild-type bulk activity (J. Blakeslee, J. Heath and A. DeLong, unpublished).

Thus our data indicate a specific requirement for the RCN1 scaffold in ACS6 dephosphorylation. These data are consistent with the specificity we observe in regulation of hypocotyl elongation; in contrast to the characteristic short hypocotyl phenotype of *rcn1* mutant plants (58.6 ± 4.1% of wild-type), *pp2aa2-1 pp2aaa3-1* seedlings exhibit nearly normal hypocotyl lengths (96.7 ± 3.6% of wild-type).

**PP2A Inhibition Reduces ACS5 Protein Accumulation**

We observed a strikingly different effect of PP2A inhibition on ACS5 protein levels. As expected, normal seedlings exhibited rapid turnover of a myc-tagged ACS5 protein (Figure 8A). In *rcn1* seedlings, myc-ACS5 accumulation was dramatically reduced (Figure 8A). Direct comparisons of myc-ACS5 abundance in serial extract dilutions indicate that myc-ACS5 accumulation is reduced at least 25-fold in *rcn1* seedlings (see Figure S2B). We observed a similar decrease in myc-ACS5 accumulation when wild-type myc-ACS5 plants were treated with cantharidin (see Figure S2D). Although the reduced baseline accumulation of ACS5 in this experiment precludes accurate measurements of turnover under conditions of phosphatase inhibition, ACS5 protein does not appear to be stabilized by the *rcn1* mutation or by cantharidin treatment, but rather appears to be de-stabilized. These experiments employed an inducible myc-ACS5 construct (Chae et al., 2003) and reduced myc-ACS5 accumulation was apparent across a wide range of induction levels (25 to 200 nM Dexamethasone; see Figure S2A). At higher levels of induction, accumulation of the myc-ACS5 protein was more

easily detected in *rcn1* mutant seedlings. Although turnover in wild-type seedlings was impeded at this expression level, myc-ACS5 was clearly unstable in the *rcn1* mutant plants (Figure S2C). Strikingly, accumulation and turnover of the stabilized myc-ACS5$^{eto2}$ protein product was not affected by the *rcn1* mutation (Figure 8B). This result argues that the decreased accumulation of wild-type myc-ACS5 results from ACS5 protein instability, rather than any effect on the Dex-inducibility of transgene expression in *rcn1* plants. It also demonstrates that PP2A-dependent regulation of ACS5 accumulation requires the C-terminal sequences that are recognized by ETO1.

These observations are consistent with the increased cantharidin responsiveness of ethylene production and hypocotyl elongation we observed in *acs5* and *acs9* mutant plants (Figure 3C and D); in type 2 *acs* mutants, baseline ACS activity levels are reduced and the relative effect of stabilizing type 1 isozymes is exaggerated. Our data suggest that loss of PP2A activity simultaneously increases accumulation and activity of type 1 isozymes while reducing accumulation and activity of type 2 isozymes. Because normal baseline ACS activity levels are very low in dark-grown seedlings, stabilization of a single isozyme type has an obvious effect on ethylene production, even when other isozymes are destabilized. In effect, the increase in ethylene production due to stabilization of type 1 isozymes masks the destabilization of type 2 enzymes in hypocotyl elongation and ethylene biosynthesis experiments, and in the *eto1* epistasis experiment.

## DISCUSSION

Low levels of ethylene biosynthesis are characteristic of etiolated *A. thaliana* seedlings, and previous work has identified protein turnover mechanisms that limit the accumulation of ACS isozymes. In conjunction with the tight regulation of *ACS* mRNA levels (Tsuchisaka and Theologis, 2004), (Guo and Ecker, 2004), these mechanisms constitute a stringent control system that regulates ethylene production (Vogel et al., 1998; Chae et al., 2003; Liu and Zhang, 2004; Wang et al., 2004; Joo et al., 2008; Kamiyoshihara et al., 2010). MAPK-mediated phosphorylation antagonizes the turnover mechanism that controls the stability of type 1 ACS isozymes in seedlings (Liu and Zhang, 2004; Schweighofer et al., 2007; Joo et al., 2008). Our data indicate that RCN1-containing PP2A complexes dephosphorylate and promote the turnover of type 1 ACS isozymes in etiolated seedlings, suggesting that PP2A-mediated protein dephosphorylation is an important counterbalance to MAPK action. Conversely, PP2A appears to positively regulate the accumulation of type 2 ACS isozymes. Thus the control systems for type 1 and type 2 isozymes are independently specialized, but both involve PP2A action (Figure 9).

Under natural conditions, down-regulation of ethylene synthesis is necessary to allow the rapid hypocotyl cell expansion that ensures the emergence of seedling shoot tissues from the soil. Ethylene overproduction in plants with reduced PP2A activity results in a characteristic short hypocotyl phenotype (Larsen and Cancel,

2003; Muday et al., 2006). Exploiting that phenotype in our genetic analysis, we found that the *ACS2* and *ACS6* genes are required, while the *ETO1*, *ACS5* and *ACS9* genes are dispensable, for increased ethylene synthesis under conditions of PP2A inhibition. Direct analysis of ethylene production in *acs* loss-of-function mutants also demonstrated the requirement for type 1 but not type 2 isozymes. ACS enzyme activity levels are increased by *rcn1* mutations and by cantharidin treatment. These results support a model in which PP2A inhibition allows accumulation of phosphorylated and stabilized type 1 ACS isozymes. Further support for this model comes from our analysis of elicitor-induced ethylene production in wild-type and *rcn1* mutant plants. Wild-type plants exhibit a dramatic increase in ethylene production after Flg22 treatment, while *rcn1* plants, in which baseline ethylene production is elevated above the wild-type level, show only a modest increase. Turnover of the wild-type ACS6 protein is retarded in *rcn1* mutant plants and in cantharidin-treated wild-type plants, while the stabilized $ACS6^{DDD}$ protein shows little or no effect of cantharidin treatment. The RCN1 protein interacts with both wild-type ACS6 and with the stabilized $ACS6^{DDD}$ protein; as might be predicted for a substrate interaction, binding to the wild-type ACS6 protein appears quite unstable. Immunoprecipitated PP2A complexes dephosphorylate a MAPK- phosphorylated ACS6 C-terminal peptide. Finally, analysis of A subunit mutants shows that the RCN1 scaffolding subunit is required for dephosphorylation of the ACS C-terminal peptide, while loss of the PP2AA2 and PP2AA3 scaffolds has only a modest effect on dephosphorylation. This specific requirement for RCN1-directed dephosphorylation in vitro is

mirrored by RCN1-specific regulation of hypocotyl elongation in vivo. Together these data suggest that PP2A complexes containing the RCN1 regulatory subunit dephosphorylate type 1 ACS isozymes, and that increased phosphorylation and stabilization of these enzymes allows increased ethylene synthesis in *rcn1* seedlings.

Recent work in tomato fruit indicates that LeACS2, a type 1 ACS isozyme of tomato, is stabilized when phosphorylated on both the CDPK and MAPK phosphorylation target sites (Kamiyoshihara et al., 2010). Treatment with a protein phosphatase inhibitor promotes the accumulation of LeACS2 that is phosphorylated at the CDPK target site, increasing ACS activity levels. (The effect of protein phosphatase inhibition on phosphorylation at the MAPK sites was not directly assayed in that work.) The effect of phosphorylation at the putative CDPK site of *A. thaliana* type 1 ACS isozymes has not yet been tested. However, substitution of phosphomimic residues in the MAPK site is sufficient to dramatically increase the stability and accumulation of ACS6, suggesting that CDPK-dependent phosphorylation is not limiting for ACS6 stability in seedlings.

The mechanism by which phosphorylation stabilizes ACS isozymes has not been clearly defined. The non-catalytic carboxy-terminal domain of ACS6 is sufficient to confer 26S-proteasome-dependent instability on GFP and luciferase reporters, and it has been suggested that this region acts as a flexible docking domain that extends from the catalytic core. The distribution of acidic and basic residues in

this region influences the degree of stabilization observed in the phosphomimicking ACS6$^{DDD}$ mutant (Joo et al., 2008), consistent with the idea that phosphorylation at both the CDPK and MAPK sites could contribute to type 1 isozyme stability. Phosphorylation at the CDPK target site in type 2 isozymes was postulated to affect interactions with the ETO1/EOL-containing E3 ubiquitin ligase complex, but non-phosphorylatable and phosphomimic alleles of *ACS5* show normal interactions with *ETO1* and its paralogs in yeast 2-hybrid assays (Christians et al., 2009), indicating that modification at this site is not sufficient to regulate this critical interaction. Binding of 14-3-3 proteins to ACS isozymes also has been detected (Chang et al., 2009) and may play a role in phosphorylation-dependent stabilization.

Unexpectedly, PP2A appears to play a positive role in regulating the accumulation of ACS5, a type 2 isozyme. Thus the net result of phosphatase inhibition on ACS activity levels in wild-type plants represents the sum of two different effects: increased accumulation of type 1 isozymes and decreased accumulation of type 2 isozymes. The *rcn1* defect dramatically reduced the accumulation of ACS5 in plants carrying an inducible transgene construct, indicating that PP2A affects some post-transcriptional mechanism required for ACS5 accumulation. Our data suggest that type 2 ACS proteins are less stable when PP2A activity is reduced, but it is unclear whether this mechanism involves direct action on type 2 isozymes or dephosphorylation of a component of the ETO1 complex (Figure 9). We have not yet determined whether ETO1 plays a

role in PP2A-mediated ACS5 stabilization. Recent proteomic profiling has identified the ETO1-like EOL1 and EOL2 proteins as well as one representative of each ACS isozyme type (ACS6, 7, 8) as 14-3-3 omega-binding clients, suggesting that these proteins are phosphorylated in vivo (Chang et al., 2009). Since type 3 isozymes do not possess a C-terminal phosphorylation motif, these data suggest that phosphorylation in the conserved catalytic domain of some isozymes also may contribute to ACS regulation. The apparent enhancement of ethylene overproduction in cantharidin-treated *acs5* and *acs9* loss-of-function mutants indicates that PP2A function affects the activity of type 2 isozymes under native expression conditions as well. Although only *ACS1* and *ACS9* were found to make statistically significant contributions to control of hypocotyl elongation in 3-day old etiolated seedlings (Tsuchisaka et al., 2009), our analysis of ethylene production shows that both *ACS5* and *ACS9* play important roles in ethylene synthesis in 5-day old seedlings, with *ACS2* and *ACS6* contributing little, when PP2A activity levels are normal. For both isozyme classes, fine-tuning of the activity levels requires protein phosphorylation/dephosphorylation and involves RCN1-regulated PP2A function.

Interestingly, when *acs2 acs6* double mutants are treated with cantharidin, ethylene production is slightly increased (Figure 3D). If overall ethylene production in *acs2 acs6* double mutants were solely dependent on type 2 ACS isozymes, we would predict that phosphatase inhibition would decrease ethylene synthesis. Phosphorylation-dependent regulation of the poorly understood type 3

isozymes may contribute to the residual cantharidin-induced ethylene synthesis observed in *acs2 acs6* double mutants. Additionally, recent analysis of single, double and multiple *acs* mutants has demonstrated that there is a complex interplay between ACS isozymes (Tsuchisaka et al., 2009), and it is possible that a compensatory mechanism is activated in *acs2 acs6* double mutants.

Earlier work suggests that *ACS* mRNA levels also are affected by reversible protein phosphorylation (Kim et al., 1997). The data reported here for accumulation of ACS5 and ACS6 proteins are derived from transgenic lines that employ constitutive (*35S::myc-ACS6*) and glucocorticoid-inducible (*myc-ACS5*) promoter fusions, and thus reflect effects of PP2A function that are independent of native *ACS* mRNA levels. Moreover, preliminary analysis of *ACS* mRNA levels suggests that *ACS6* transcript levels are normal, while *ACS5* and *ACS9* transcript levels increase, in *rcn1* mutant plants (M. Soruco and A. DeLong, unpublished). Since our genetic analysis indicates that ethylene overproduction requires *ACS2* and *ACS6*, but not *ACS5* and *ACS9*, these results suggest that effects on mRNA accumulation do not account for the ethylene overproduction phenotype of *rcn1* mutant seedlings. Similarly, analysis of enhanced LeACS2 accumulation after phosphatase inhibitor treatment indicates that mRNA levels remain unchanged while protein stability is increased (Kamiyoshihara et al., 2010).

## MATERIALS AND METHODS

### Plant Material

To generate dark-grown seedlings for physiological and biochemical analysis, *A. thaliana* seeds were surface-sterilized, suspended in 0.1% agar and stratified for 3 days at 4°C before plating. Seedlings were grown on 0.5X Murashige and Skoog (MS) salts with 1% sucrose and 1% agar. After sowing, seeds were given a 16-hour light treatment before transfer into the dark for germination and growth at 24°C on vertical plates. Hypocotyl elongation, ACS activity and protein accumulation phenotypes were scored 5 days post germination. For hypocotyl elongation assays, approximately 30 seeds were sown on each plate; at the conclusion of the growth period, plates were imaged on a flatbed scanner. Hypocotyl lengths were measured using Image J. Cantharidin responsiveness of hypocotyl elongation was assayed in four separate experiments; results shown in Figure 3 represent the aggregate values ± standard error.

The *rcn1-1* mutant (Garbers et al., 1996) and the *rcn1-1* RCN1-YFP transgenic line (Blakeslee et al., 2008) are in the Wassilewskija (Ws) genetic background. The *rcn1-6* mutant (Blakeslee et al., 2008) and all other mutants and transgenic lines used in this work are in the Columbia (Col) genetic background. Transgenic 35S::myc-ACS6, 35S::myc-ACS6$^{DDD}$ and the *acs2* and *acs6* mutant lines (Joo et al., 2008) as well as the *acs2 acs6* double mutant (Han et al., 2010) were the kind gift of S. Zhang (University of Missouri, Columbia). In *acs* loss-of-function

experiments, we also used the *acs5-3* (*cin5*) and *acs9-1* alleles (Vogel et al., 1998; Tsuchisaka and Theologis, 2004). Crossing *rcn1-1* and *eto1-1* generated the *rcn1 eto1* double mutant in a mixed Ws/Col background. Double mutant F3 families were compared to the parental single mutants and to single mutant sibling families segregating out of the cross.

**ACS Activity Assays**

For ACS enzymatic activity assays, etiolated seedlings were harvested and ground in liquid nitrogen, resuspended in ACS protein extraction buffer (Liu and Zhang, 2004) and assayed for ACS activity as described previously (Chae et al., 2003). After chemical conversion of ACC to ethylene, 750 µl of headspace was transferred to a new vial for analysis on a Voyager portable gas chromatograph (PhotoVac Inc.). All reactions were carried out in triplicate.

**Protein Turnover Assays**

For transgenic 35S::myc-ACS6 lines, MG-132 pre-treatment was used to promote protein accumulation (Joo et al., 2008). For transgenic myc-ACS5 lines, seedlings were grown in the presence of low concentrations of Dexamethasone as previously described (Chae et al., 2003). At the beginning of each turnover assay, seedlings were washed 3 times in liquid MS medium for 5 minutes and resuspended in liquid MS containing 1 mM cycloheximide. Samples were harvested and flash-frozen in liquid nitrogen in the dark at the specified time

points and stored at -80°C until ACS stability was analyzed via immunoblotting with anti-myc antibody.

**Immunoblotting and Immunoprecipitation**

For immunoblotting experiments, seedlings were ground to a fine powder in liquid nitrogen and boiled for 10 minutes with 4X SDS loading buffer (240 mM Tris pH 6.8, 8% SDS, 40% glycerol, 0.04% bromophenol blue, 5% beta-mercaptoethanol). Extracts were centrifuged at 16,000 X g for 15 minutes at 4°C and supernatants were harvested for immediate use or storage at -20°C. Extracts were separated by electrophoresis on a 10% SDS-polyacrylamide gel, then transferred to a PVDF membrane (Millipore). Detection of proteins was performed using monoclonal anti-myc 9E10 (Covance), antisera against phospho-enol pyruvate carboxylase (anti-PEPC, Rockland) or polyclonal anti-RCN1 antibodies (Deruère et al., 1999) and standard chemiluminescence.

For immunoprecipitation of PP2A complexes, RCN1-YFP seedlings were grown in the dark for 5 days on MS plates. Seedlings were then harvested and ground in liquid nitrogen, thawed in co-IP buffer (50 mM Tris, pH 7.5, 100 mM NaCl, 0.3 M sucrose, 0.2% Triton X-100, 2 µg/ml aprotinin and leupeptin). Extracts were centrifuged for 15 minutes at 16,000 X g at 4°C to pellet debris, and the protein concentration of the supernatant was adjusted to 1.67 mg/ml with ice-cold co-IP buffer. For each precipitation, 250 µg of protein extract was incubated with 200 µl Protein A agarose (Invitrogen) plus 50 µl of 1.5:100 dilution of anti-GFP

antibody (AbCam) for 1 hour at 4°C. Immunoprecipitates were harvested by centrifugation at 1,000 X g for 3 minutes at 4°C and washed twice in ice-cold co-IP buffer. For peptide dephosphorylation experiments, immunoprecipitates were resuspended in 200 µl of PPAB and aliquots were added to dephosphorylation reactions. For co-immunoprecipitation of ACS6 and PP2A, myc-ACS6 and myc-ACS6$^{DDD}$ seedlings were grown in the dark on MS plates for 4.5 days and then transferred to liquid MS media containing MG-132 for 16 hours to allow ACS protein accumulation [10]. Protein extracts were prepared and processed with anti-myc or anti-RCN1 antibodies as described above, and immunoprecipitates were eluted from the Protein A agarose with SDS loading buffer, boiled and analyzed by immunoblotting as described above.

**Phosphatase Treatment of myc-ACS6**

Protein extracts (62.5 µg total protein) from MG-132 treated myc-ACS6 seedlings were treated for 10 minutes at 37° with a phosphatase or with extraction buffer (100 mM HEPES, pH 7.5, 5 mM EDTA, 5 mM EGTA, 1 mM PMSF, 2 mM benzamidine, 2 µg/ml aprotinin and leupeptin) alone. For CIP treatment, 5 units of alkaline phosphatase (CIP, New England Biolabs) were added. For PP2A treatment, anti-GFP antibodies were used to immunoprecipitate PP2A complexes from seedlings expressing the RCN1-YFP fusion as described above; 10 µl of PP2A immunocomplexes were added to the myc-ACS6 extract. After phosphatase treatment, samples were boiled in SDS loading buffer and the migration of myc-ACS6 was analyzed by immunoblotting.

**Peptide Dephosphorylation Assays**

Recombinant His-tagged $MKK4^{DD}$ and MPK6 were purified using Ni-NTA affinity chromatography (Liu and Zhang, 2004) and used to phosphorylate 500 µg of a biotinylated ACS6 peptide comprising the 30 C-terminal amino acids of ACS6 (generous gift of S. Zhang, University of Missouri, Columbia) in a buffer containing 200 µM ATP plus 12 µCi $\gamma$-$^{33}$-P-ATP. Radiolabeled peptide (15 pmol/well) was then bound to a streptavidin-coated 96-well plate (Thermo Scientific) and incubated with shaking at 4°C for 2 hours. Each well was washed 4 times for 5 minutes with PPAB (50 mM Tris-HCl pH 7.0, 0.1 mM EDTA, 5 mM DTT, 0.01% Brij-35). For immunocomplex assays, PP2A complexes were isolated from plants expressing an RCN1-YFP fusion protein (Blakeslee et al., 2008) using either a polyclonal anti-GFP antibody (AbCam) or Protein A agarose alone. After a 60-minute immunoprecipitation and two stringent washes at 4°C, IP pellets were resuspended in PPAB and 20 µl aliquots were assayed for ACS6 dephosphorylation activity (4 replicate reactions per IP pellet). After 15 minutes at 30°C, reactions were stopped with 4X loading buffer and each supernatant was sampled for released counts. Immunoprecipitation fractions also were subjected to immunoblot analysis using anti-RCN1 and anti-C subunit antibodies to confirm isolation of PP2A immunocomplexes. For crude extract assays, dark-grown seedlings were ground in liquid nitrogen and resuspended in PPAB. Extracts were diluted to a protein concentration of 2.5 µg/ml in PPAB containing okadaic acid at final concentrations of 0, 1 or 1000 nM, and 50 µl aliquots were

added to 96-well plates pre-bound with 0.25 µg phospho-ACS6 peptide. Triplicate reactions were incubated at 30°C for 15 minutes before termination by addition of 4X loading buffer. Each supernatant was sampled for released counts, and the background activity observed at 1000 nM OKA was subtracted from the average values obtained in the presence of 0 and 1 nM OKA.

**Ethylene Evolution Measurements**

For analysis of the effect of cantharidin on ethylene biosynthesis, seedlings were grown on 3 ml MS medium containing 3 µM cantharidin or a DMSO vehicle control in 22-mL gas chromatography vials for 5 days at 23°C in the dark. For Flg22 induction, seedlings were grown in long days at 23°C in 22-mL gas chromatography vials containing 3 ml MS medium for 12 days. Flg22 peptide (final concentration 40 µM) was added at day 12 and the vials were immediately capped and further incubated 4 hrs at 23°C in the light. In both experiments, the accumulated ethylene was measured by gas chromatography as described previously (Vogel et al., 1998). Flg22 peptide was the kind gift of S. Zhang, University of Missouri, Columbia.

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## FIGURES

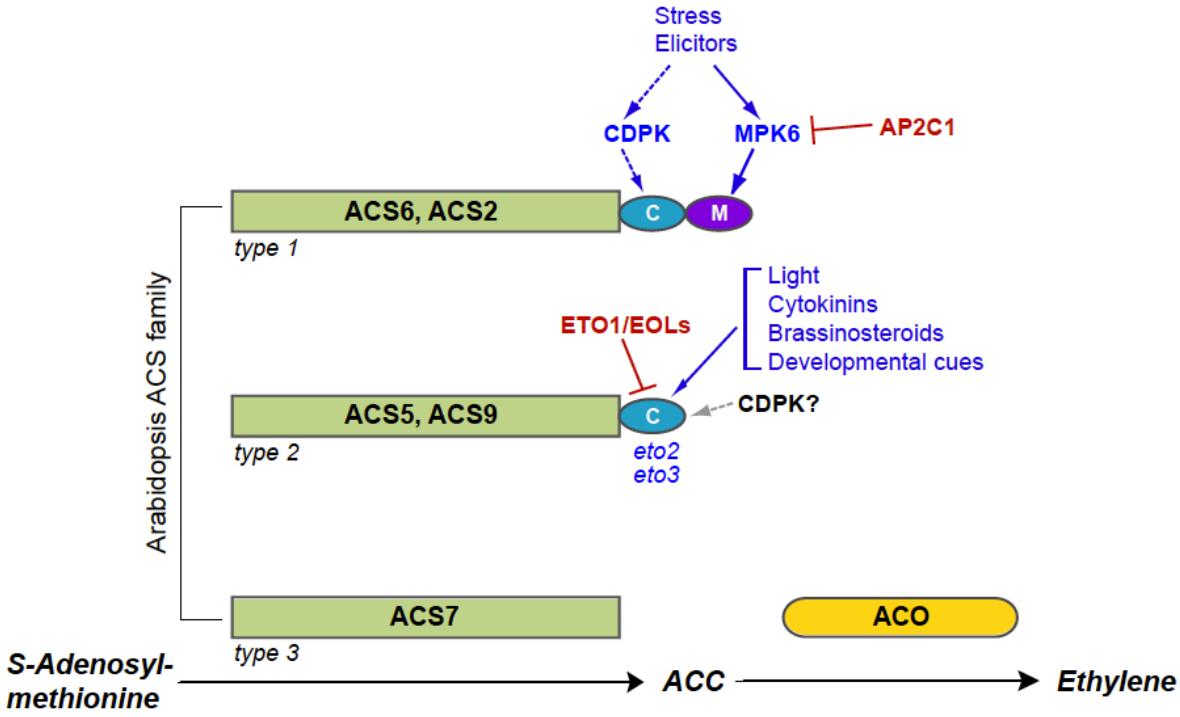


**Figure 1. Post-translational modifications of ACS proteins regulate ethylene synthesis.** The three ACS isozyme types are defined by their C-terminal amino acid sequence motifs; representative members of each type are shown. Consensus sites for MAPK and CDPK phosphorylation are shown as purple and turquoise ovals, respectively. Several factors and signals that affect the stability of type 1 and type 2 isozymes through action on C-terminal motifs are indicated, with positive regulators shown in blue and negative regulators shown in red. In the absence of a stabilizing input, type 1 and type 2 isozymes are rapidly degraded by the 26S proteasome. Evidence for CDPK regulation of type 1 isozymes has been obtained in tomato fruits; dashed lines indicate that this role has not yet been established in Arabidopsis. Although the C-terminus of type 2 isozymes can be phosphorylated by CDPKs in vitro, a regulatory role for CDPK phosphorylation of these proteins has not been demonstrated in vivo; the gray arrow indicates possible CDPK input. The *eto2* and *eto3* mutations are stabilizing alleles of ACS5 and ACS9, respectively. Not depicted in this cartoon are ACS4 and ACS8, two additional type 2 isozymes, and ACS1, a catalytically inactive type 1 isozyme.

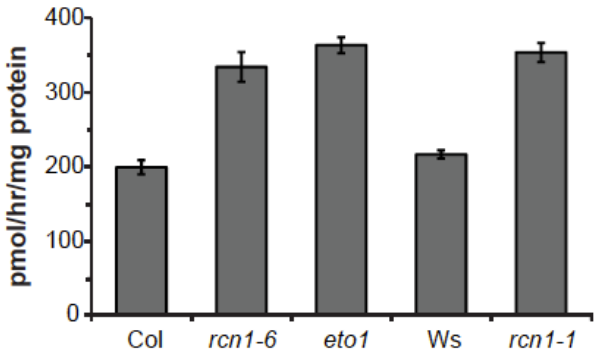


**Figure 2. ACS enzymatic activity is increased in *rcn1* mutant seedlings.** Total seedling proteins were extracted from etiolated wild-type and mutant plants five days post-germination. ACS enzymatic activities were assayed in triplicate (see Materials and Methods).

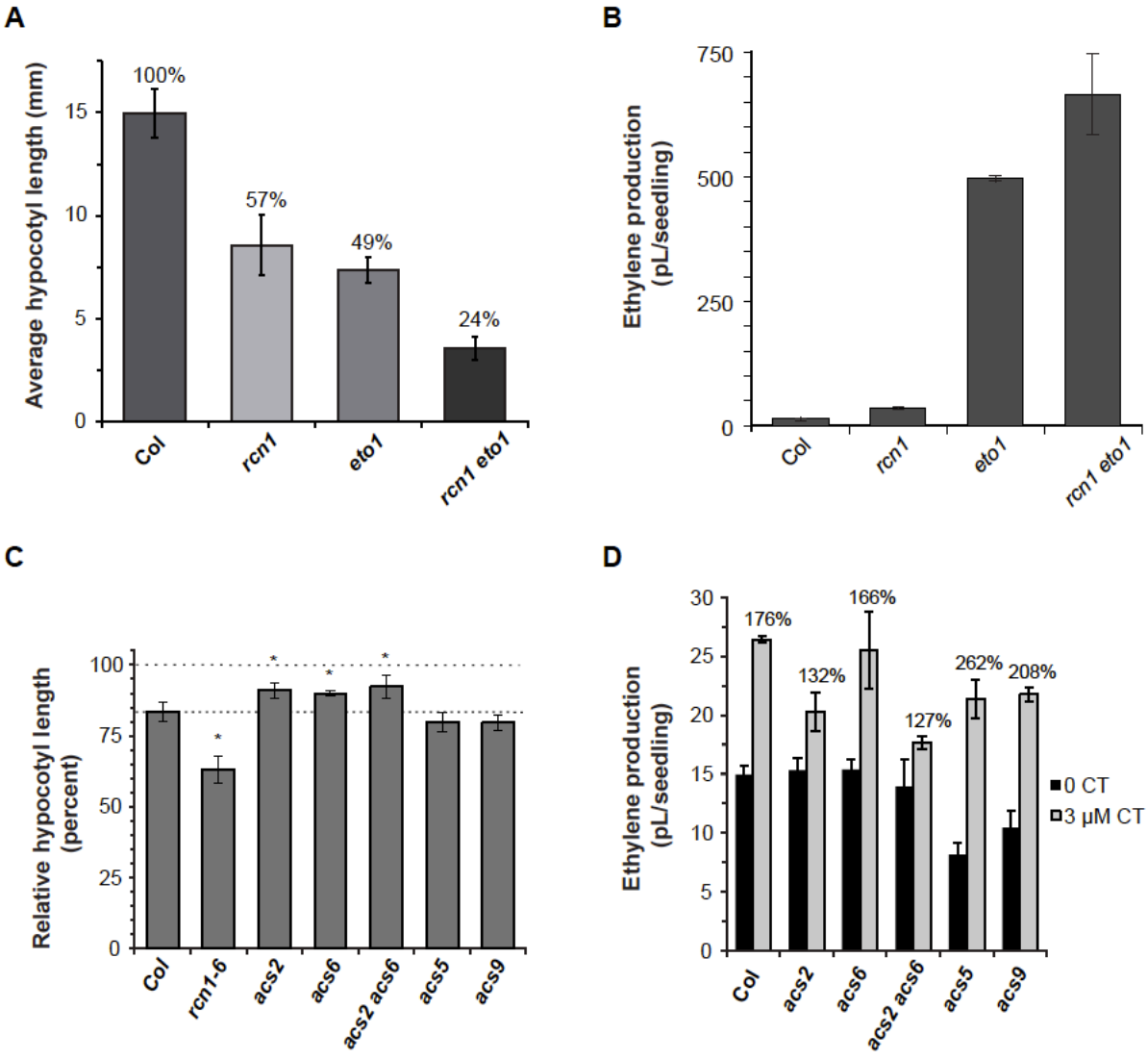


**Figure 3. PP2A-dependent ethylene overproduction is *ETO1*-independent and requires type 1 isozymes**. (A) The hypocotyl elongation defects of the *rcn1-1* and *eto1-1* single mutants are additive in the *rcn1 eto1* double mutant. Hypocotyl lengths of wild-type and mutant seedlings were measured after 5 days growth in the dark. Values shown represent average lengths; error bars show standard deviation (n > 25). Shading differences indicate statistically significant differences in hypocotyl length ($p < 0.001$). (B) The ethylene overproduction defects of the *rcn1-1* and *eto1-1* single mutants are additive in the *rcn1 eto1* double mutant. Wild-type and mutant seedlings were grown in sealed vials and ethylene levels were measured after 5 days growth in the dark. Values shown represent the average ethylene volume released per seedling. Error bars represent standard deviation (n ≥ 4). (C) The cantharidin responsiveness of hypocotyl growth is reduced in plants carrying *acs2* and *acs6* mutations. Wild-type and mutant seedlings were grown in the presence or absence of 3 µM cantharidin, and hypocotyl lengths were measured after 5 days growth in the

dark. Values shown represent average hypocotyl lengths of CT-grown plants as a percentage of the control length for that genotype; error bars show standard error (see Materials and Methods). Asterisks indicate a statistically significant difference in cantharidin response ($p < 0.05$). (**D**) Cantharidin-induced ethylene synthesis is reduced in *acs2* and *acs2 acs6* mutants. Wild-type and mutant seedlings were grown in sealed vials in the presence or absence of 3 μM cantharidin and ethylene levels were measured as described above; the percent increase induced by cantharidin treatment is shown for each genotype. Error bars represent standard deviation ($n = 3$).

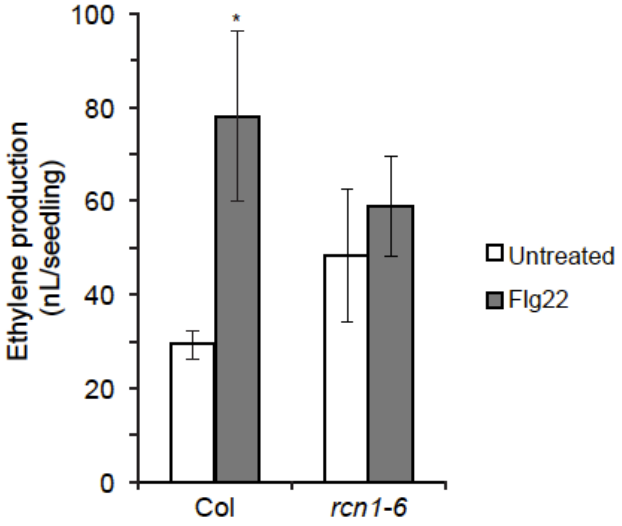


**Figure 4. Flg22-induced ethylene production is reduced in *rcn1* seedlings.** Wild-type and mutant seedlings were grown for 12 days in the light, and ethylene produced during a four-hour Flg22 treatment was measured (see Materials and Methods). Each value represents the average volume of ethylene released per seedling (n = 3). Error bars indicate standard deviation. Flg22 treatment induces a significant increase (*) only in wild-type Col seedlings ($p < 0.05$).

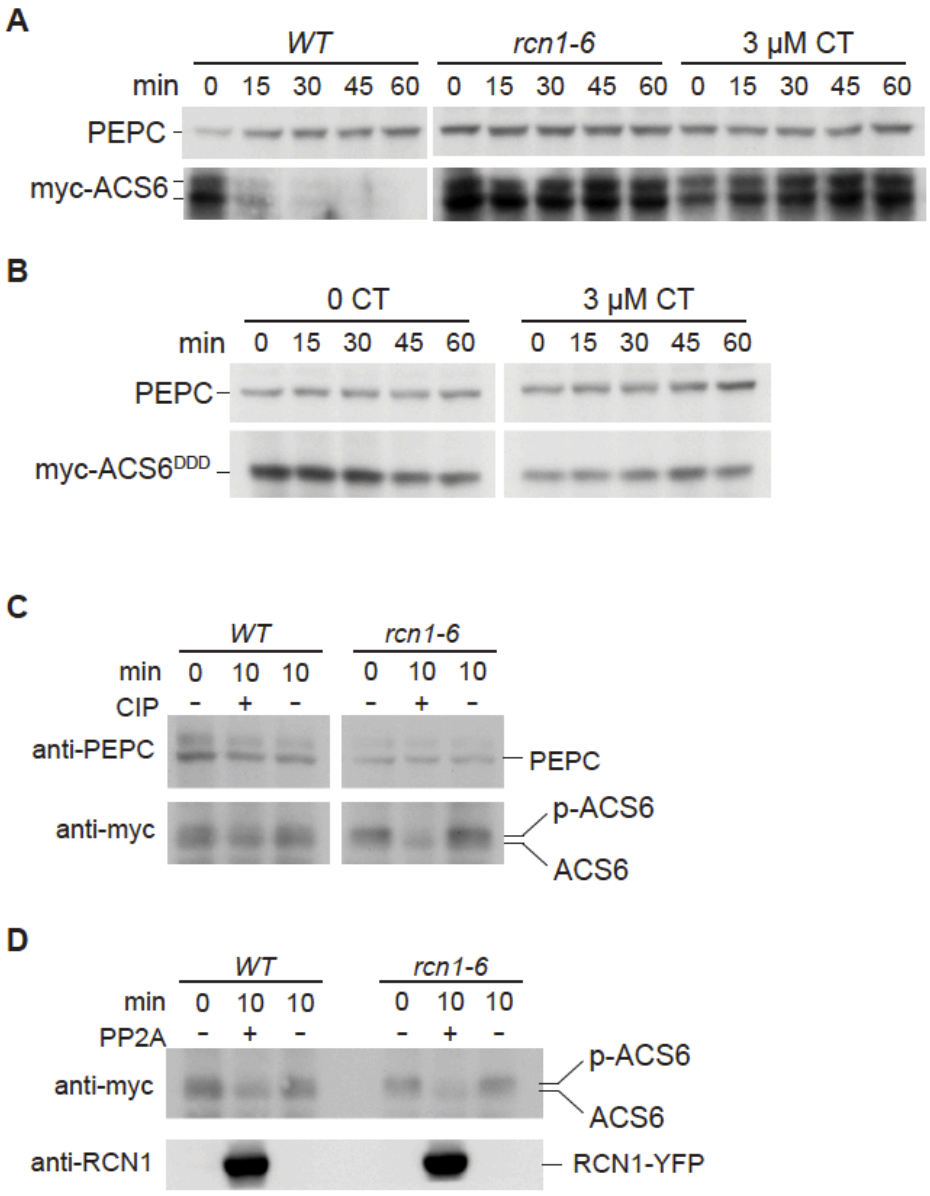


**Figure 5. PP2A inhibition stabilizes ACS6 protein.** Wild-type and *rcn1-6* seedlings expressing a wild-type *myc-ACS6* transgene (A) or a stabilized *myc-ACS6$^{DDD}$* transgene (B) were grown in the absence or presence of 3µM cantharidin (CT), and ACS6 turnover was assayed by immunoblotting extracts harvested at time points after addition of the protein synthesis inhibitor cycloheximide (see Materials and Methods). Immunoblots were probed with anti-myc to detect myc-ACS6 turnover and anti-PEPC as a loading control. (C) Extracts from wild-type and *rcn1-6* seedlings expressing myc-ACS6 were subjected to calf intestinal alkaline phosphatase (CIP) treatment (+) or incubated in buffer without CIP (-) for ten minutes. Samples were separated by SDS-PAGE to resolve the phosphorylated (P-ACS6) and dephosphorylated (ACS6) myc-

ACS6 bands (see Materials and Methods). Immunoblots were probed with anti-PEPC (upper panel) as a loading control and with anti-myc (lower panel) to detect myc-ACS6. Migration of myc-ACS6 in treated samples can be compared to myc-ACS6 in untreated extracts (0 min) that were immediately boiled in SDS loading buffer. (D) Extracts from wild-type and *rcn1-6* seedlings expressing the wild-type myc-ACS6 were subjected to treatment with PP2A complexes (PP2A) immunoprecipitated from plants expressing RCN1-YFP (+), or incubated in buffer alone (-) for 10 minutes. Samples were processed as described for CIP-treated extracts (panel C above). Immunoblots were probed with anti-myc (upper panel) to detect myc-ACS6 and with anti-RCN1 (lower panel) to detect the RCN1-YFP fusion protein in PP2A-treated samples.

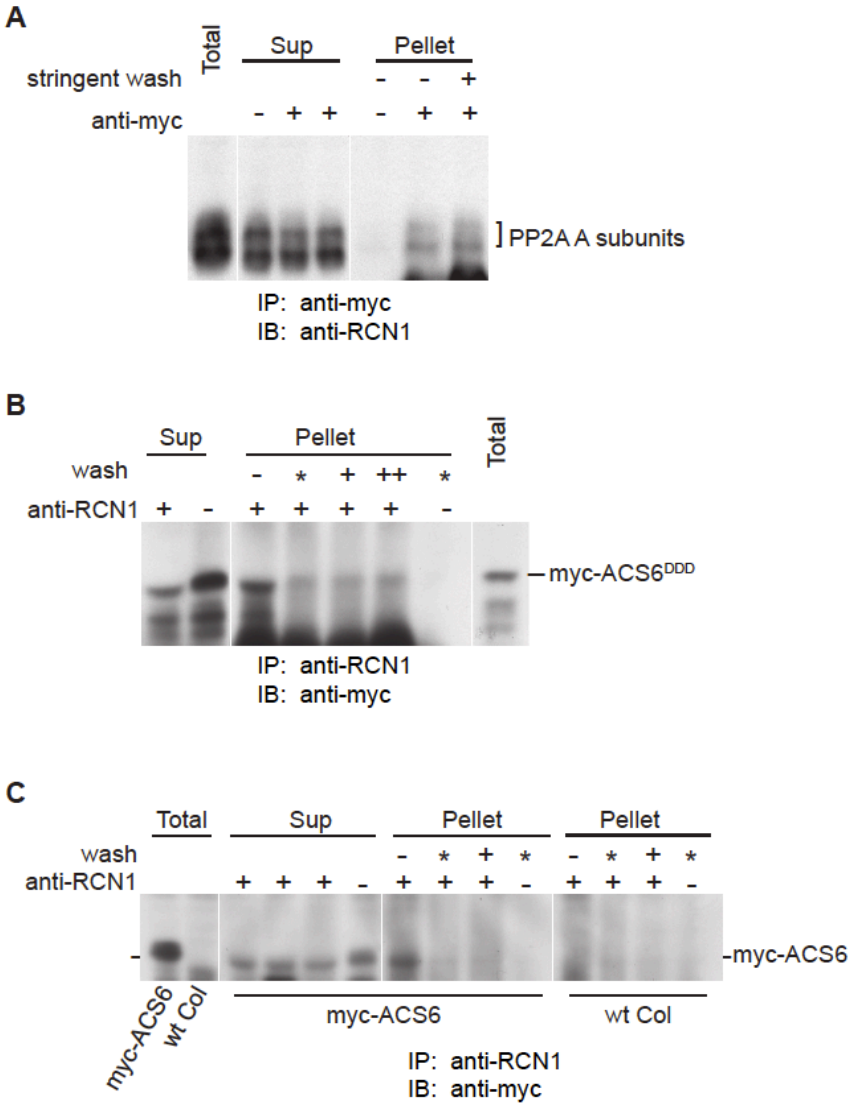


**Figure 6. PP2A interacts with ACS6 in reciprocal co-immunoprecipitations.** Protein extracts from MG132-treated seedlings expressing myc-ACS6$^{DDD}$ (A, B) or wild-type myc-ACS6 (C) were subjected to immunoprecipitation (IP) with anti-myc (A) or anti-RCN1 (B, C) antibodies followed by immunoblotting (IB) with the antibodies indicated. Immunoprecipitates (Pellet fractions) were isolated without a wash (-), or were washed with a detergent-free buffer (*), with buffer containing 0.2% Tween-20 (+), or with buffer containing 0.2% Tween-20 plus 0.1% Triton X-100 (++). Samples of the supernatant fractions (Sup) were also analyzed to test for depletion of the putative interactor. Control immunoprecipitates (without anti-myc or anti-RCN1 antibodies) were washed with detergent-free buffer. Because the signal for wild-type myc-ACS6 was very weak in anti-RCN1 immunoprecipitations (C), extracts from wild-type Col plants lacking a *myc-ACS6* transgene were used as a control for background signals.

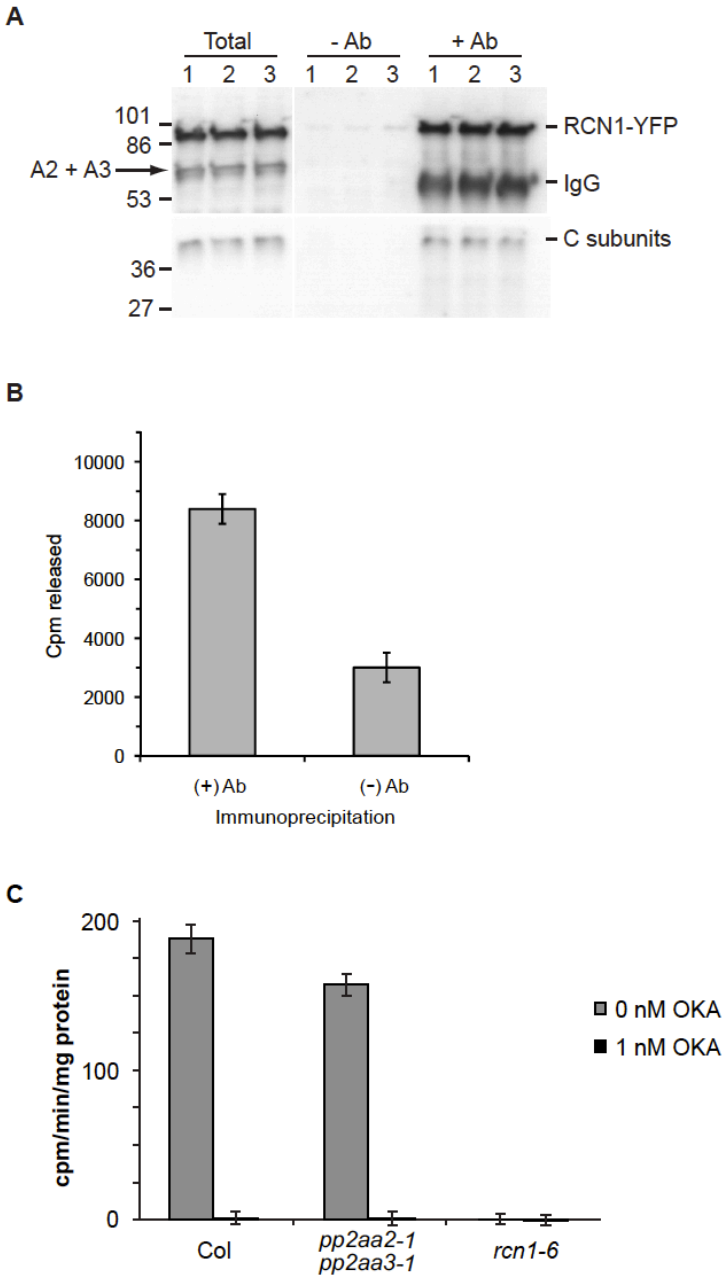


**Figure 7. RCN1-containing PP2A complexes dephosphorylate the ACS6 C-terminal phosphopeptide**. PP2A immunocomplexes were isolated and assayed for peptide dephosphorylating activity (A, B). Total protein extracts were prepared from three independent samples of dark-grown seedlings expressing an RCN1-YFP fusion protein (Blakeslee et al., 2008). Each extract was divided and immunocomplexes were isolated using anti-GFP antibodies (+Ab) or Protein A agarose beads alone (-Ab). Fractions of the total extracts (Total) and the immunoprecipitated pellets (-Ab and +Ab) were subjected to SDS-PAGE and immunoblotting (A) using anti-RCN1 (upper panel) and anti-C subunit (lower panel) antisera. Anti-RCN1 antibodies detect both the RCN1-YFP fusion protein

and the PP2AA2 and PP2AA3 (A2 + A3) proteins. (B) The (+) Ab and (-) Ab immunocomplexes were assayed for phosphatase activity against a $^{33}$P-labeled ACS6 phosphopeptide (see Materials and Methods). Values shown represent the average dephosphorylating activities present in the immunocomplexes. Error bars show standard error. Average dephosphorylation activities of (+) Ab and (-) Ab pellets are significantly different ($p < 0.05$). (C) Crude extracts from wild-type and from *rcn1-6* and *pp2aa2-1 pp2aa3-1* mutant seedlings were assayed for $^{33}$P-labeled ACS6 phosphopeptide phosphatase activity in the presence or absence of 1 nM okadaic acid (OKA), which specifically inhibits PP2A (see Materials and Methods). Values shown represent the average peptide dephosphorylation activities. Error bars show standard deviation.

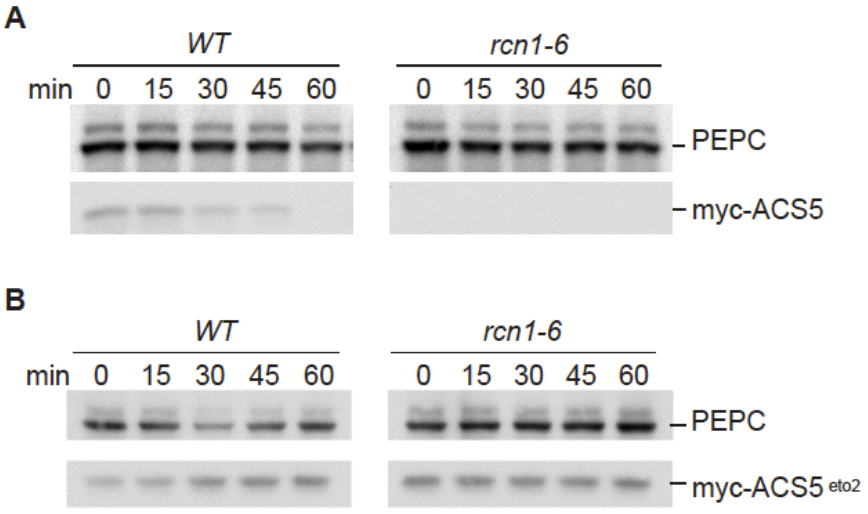


**Figure 8. PP2A inhibition decreases ACS5 accumulation.** Wild-type and *rcn1-6* mutant seedlings carrying Dexamethasone-inducible wild-type *myc-ACS5* (A) or *myc-ACS5*$^{eto2}$ (B) transgenes were grown on media containing 25 nM Dexamethasone and extracts were harvested at time points after addition of the protein synthesis inhibitor cycloheximide. Immunoblots were probed with anti-myc to detect myc-ACS5 proteins and with anti-PEPC as a loading control.

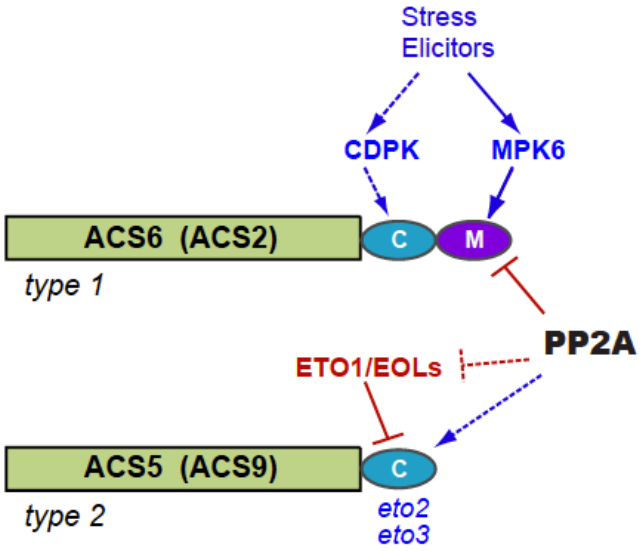


**Figure 9. Model for PP2A-mediated regulation of ACS isozyme stability**. Our results suggest that RCN1-containing PP2A destabilizes ACS6 through direct dephosphorylation of the C-terminus. This dephosphorylation opposes stabilizing C-terminal phosphorylation catalyzed by MPK6 (and possibly CDPK). Conversely, PP2A has a stabilizing effect on ACS5. Stabilization requires the wild-type C-terminal ACS5 sequence, and could involve direct action on type 2 ACS isozymes and/or inhibition of ETO1/EOL protein function. These possible modes of action are represented by dotted lines. Our genetic data indicate that PP2A-mediated regulation of ACS2 and ACS9 parallels that observed for ACS6 and ACS5, respectively.

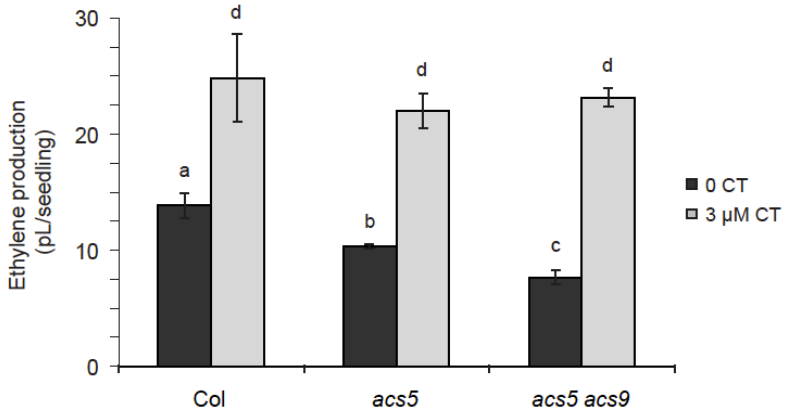


**Figure S1. Increased cantharidin responsiveness in *acs5 acs9* ethylene production.** Wild-type, *acs5* and *acs5 acs9* seedlings were grown in sealed vials in the presence or absence of 3 µM cantharidin and ethylene levels were measured after 5 days growth in the dark. Each value shown represents the average amount of ethylene released per seedling; the percent increase induced by cantharidin treatment is shown for each genotype. Error bars represent standard deviation (n = 3). Different letters indicate significant differences ($p < 0.05$).

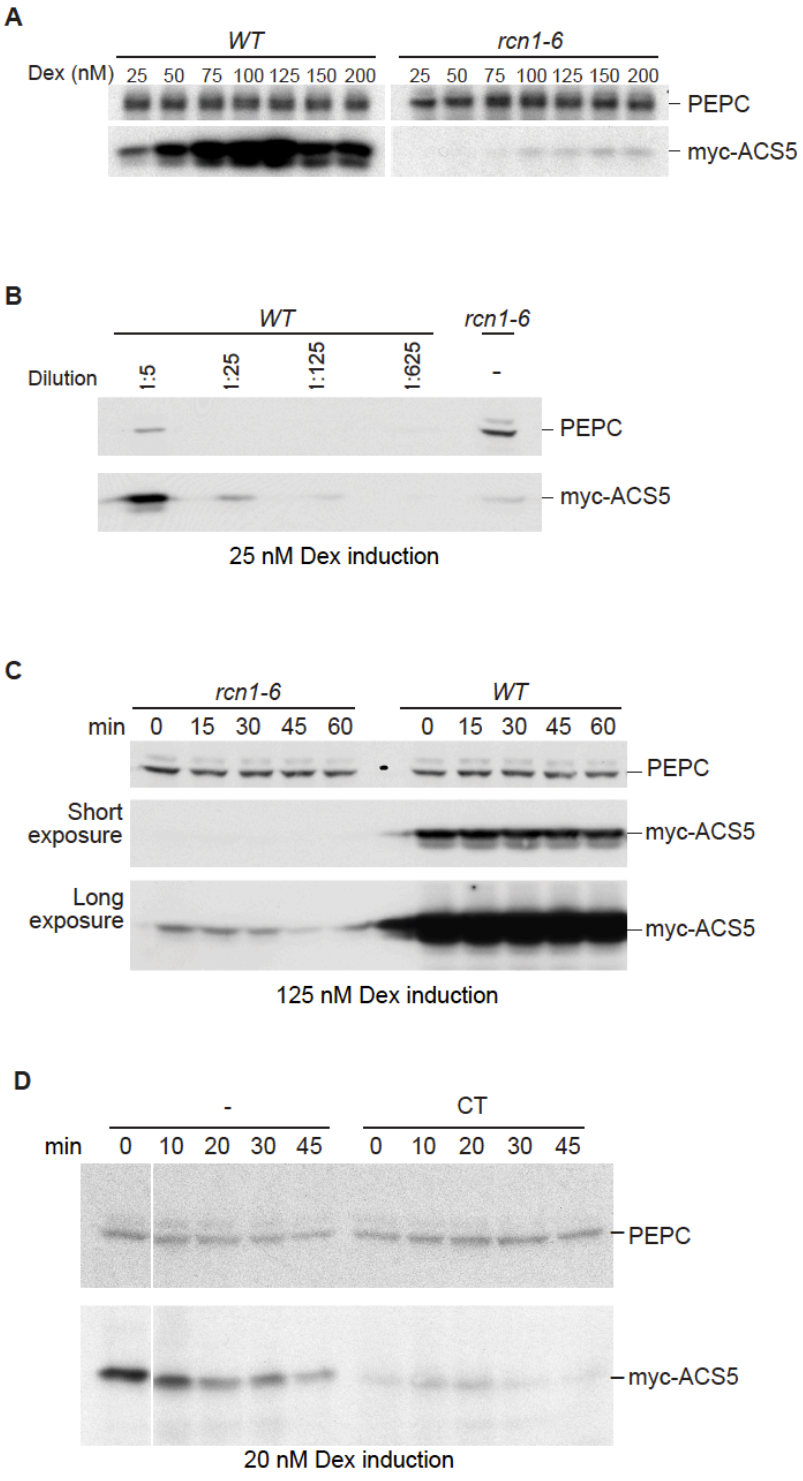


**Figure S2. The *rcn1-6* mutation reduces myc-ACS5 stability and accumulation.** (A) Wild-type and *rcn1-6* seedlings carrying a Dexamethasone-inducible wild-type *myc-ACS5* transgene were grown in the dark on medium containing Dexamethasone at the concentrations indicated (nM). Seedlings were harvested and the abundance of myc-ACS5 in total protein extracts was assayed

by immunoblotting with anti-myc and with anti-PEPC (loading control). (B) Extracts from wild-type plants were serially diluted in five-fold steps and subjected to immunoblotting to allow comparison of the abundance of myc-ACS5 in extracts from wild-type and *rcn1-6* seedlings grown on 25 nM Dexamethasone. Blots were probed with anti-myc and with anti-PEPC. (C) Wild-type and *rcn1-6* seedlings carrying *myc-ACS5* were grown on medium containing 125 nM Dexamethasone and extracts were harvested for immunoblotting at time points after addition of the protein synthesis inhibitor cycloheximide. Blots were probed with anti-myc to detect myc-ACS5 turnover and with anti-PEPC as a loading control. The short exposure of the anti-myc blot allows visualization of myc-ACS5 from wild-type samples, while the longer exposure shows protein turnover in *rcn1-6* seedlings. (D) Seedlings carrying *myc-ACS5* were grown on medium containing 20 nM Dexamethasone in the absence (-) or presence (CT) of 3 μM cantharidin and extracts were harvested for immunoblotting at time points after addition of the protein synthesis inhibitor cycloheximide. Blots were probed with anti-myc to detect myc-ACS5 turnover and with anti-PEPC as a loading control.

# CHAPTER 4 – CONCLUSIONS AND FUTURE DIRECTIONS

My analysis of Arabidopsis PP2A-A subunit functions and the role of PP2A in regulating ethylene biosynthesis provide insight into the *in vivo* functions of PP2A in seedlings. Reduced PP2A activity in *rcn1* seedlings results in pleiotropic defects. Characteristic *rcn1* defects include increased sensitivity to stress, altered root morphology, increased basipetal auxin transport, and increased ethylene production and a short hypocotyl in etiolated seedlings. Rescue of these phenotypes using transgenic plants harboring PP2A-A promoter-coding sequence swap transgenes uncovered the PP2A-A genetic requirements for proper function. In hypocotyl tissue, rescue of normal cell expansion in *rcn1* seedlings by *PP2AA3* expression demonstrates overlapping function of the PP2A-A subunit isoforms. However, *RCN1* is required for maintaining normal root stem cell organization, as expression of *PP2AA3* under control of the *RCN1* promoter does not fully rescue normal cell division patterns. These data support the general hypothesis that root growth is regulated by PP2A substrates specifically targeted by RCN1, while targeting of substrates involved in hypocotyl growth is not dependent on a particular A subunit isoform.

In the hypocotyl, low levels of ethylene biosynthesis allow for rapid cell expansion in etiolated seedlings. My analysis of PP2A-regulated ethylene production in etiolated seedlings indicates that PP2A complexes dephosphorylate and promote the turnover of type 1 ACS isozymes, permitting the maintenance of low levels of ethylene production. Using a genetic analysis, I identified that *ACS2* and *ACS6* are required for ethylene overproduction under PP2A-limiting conditions, while

*ETO1*, *ACS5* and *ACS9* are dispensable. Direct analysis of ethylene production in *acs* loss-of-function mutants also demonstrated the requirement for type 1 but not type 2 isozymes. ACS enzyme activity is increased in *rcn1* seedlings and by cantharidin treatment. These results support a model in which PP2A inhibition allows accumulation of phosphorylated and stabilized type 1 ACS isozymes. Proteolytic turnover of the wild-type ACS6 protein is retarded in *rcn1* seedlings and in cantharidin-treated wild-type plants, while the phosphomimic ACS6$^{DDD}$ protein shows little or no effect of cantharidin treatment. Additionally, RCN1 interacts with both wild-type ACS6 and with the stabilized ACS6$^{DDD}$ protein in co-immunoprecipitation assays. Further supporting an enzyme-substrate relationship, immunoprecipitated PP2A complexes dephosphorylate a MAPK-phosphorylated ACS6 C-terminal peptide. Finally, analysis of A subunit mutants shows that the RCN1 scaffolding subunit is required for dephosphorylation of the ACS C-terminal peptide, while loss of the PP2AA2 and PP2AA3 scaffolds has only a modest effect on dephosphorylation. This specific requirement for RCN1-directed dephosphorylation *in vitro* is mirrored by RCN1-specific regulation of hypocotyl elongation *in vivo*. Together these data suggest that PP2A complexes dephosphorylate type 1 ACS isozymes, and that increased phosphorylation and stabilization of these enzymes allows increased ethylene synthesis in *rcn1* seedlings.

Interestingly, PP2A appears to positively regulate the accumulation of type 2 ACS isozymes. Analysis of *acs5*, *acs9*, and *acs5acs9* seedlings demonstrated

that these type 2 ACS mutants show increased sensitivity to cantharidin in both hypocotyl elongation assays and ethylene evolution experiments. Further analysis has also demonstrated that PP2A complexes interact with both the wild type ACS5 and ACS5$^{eto2}$. Thus the control systems for type 1 and type 2 isozymes are independently specialized, but both involve PP2A action.

**The Challenges of Identifying PP2A Holoenzyme-Substrate Interactions**

The diverse composition of PP2A holoenzymes and the fact that PP2A is required for viability have made the identification specific PP2A holoenzyme complexes responsible for the dephosphorylation of defined phosphosubstrates difficult (DeLong, 2006). The use of sophisticated proteomic, genetic, and biochemical analyses has led to the identification of several specific PP2A-regulated dephosphorylation events in animals. Inhibitor studies in animals have implicated PP2A in the regulation of cell growth and proliferation (Sontag, 2001). PP2A controls cell growth through several distinct pathways and recent work has provided molecular insight into some of these PP2A-regulated events. Notable examples include the finding that PP2A$^{B'}$ holoenzymes dephosphorylate ERK in human cell lines (Letourneux et al., 2006), that PP2A$^{B\text{-alpha}}$ holoenzymes dephosphorylate AKT at Thr308 in human cell lines (Kuo et al., 2008), and that PP2A$^{B'}$ holoenzymes counteract phosphorylation of the insulin pathway kinase S6K in Drosophila (Hahn et al., 2010). Although recent progress has been made identifying plant PP2A holoenzyme-specific phosphosubstrates (see below), there were no specific phosphosubstrate-PP2A regulatory events that had been

identified at the time I began my Ph.D. thesis research. One of the major goals of my graduate work has been to identify *in vivo* PP2A targets.

**The Utility of *rcn1* as a (partial) PP2A Loss-Of-Function Mutant**

Although many animal genomes encode two isoforms of PP2A-A ($\alpha$ and $\beta$), the Arabidopsis genome encodes three PP2A-A isoforms, *RCN1, PP2AA2,* and *PP2AA3* (Zhou et al., 2003; Zhou et al., 2004). This may even be unusual in the plant kingdom as the rice (*Oryza sativa*) genome encodes only one PP2A-A scaffold, RPA1 (Yu et al., 2001; Blakeslee and DeLong, unpublished data). Studies in yeast have demonstrated that PP2A-A is a positive regulator of PP2A activity. Strains lacking the sole PP2A-A subunit (*tpd3*$\Delta$), exhibit severely retarded growth associated with an approximate 70% decrease in bulk PP2A activity, as measured by dephosphorylation of the model PP2A substrate phosphorylase *a* (Hombauer et al., 2007). Interestingly, in Arabidopsis, RCN1 functions as the primary PP2A-A subunit positively regulating PP2A activity (Zhou et al., 2004). Loss-of-function *rcn1* mutant plants exhibit pleiotropic defects associated with an approximate 2-fold drop in bulk PP2A activity (Deruere et al., 1999). In contrast, *pp2aa3* and *pp2aa3* loss-of-function mutants appear phenotypically normal (Zhou et al., 2004). As the defects apparent in the *rcn1* mutant can be phenotypically mimicked by treatment with a low dose of the selective PP2A inhibitor, cantharidin, we assume that most (if not all) *rcn1* defects result from reduced PP2A activity (Deruere et al., 1999; Zhou et al., 2004; Blakeslee et al., 2008). Therefore we hypothesize that *rcn1* phenotypes

are the result of hyperphosphorylation of normal PP2A targets and that comparative analysis of wild-type and *rcn1* plants provides us with a useful method to identify *in vivo* PP2A substrates. Furthermore, mutant phenotypes should be linked to specific hyperphosphorylated targets.

Interestingly, decreased PP2A activity in *rcn1* seedlings could result from at least three distinct molecular mechanisms. First, PP2A-A subunit isoforms direct assembly of specific PP2A holoenzymes through preferential interactions with some regulatory PP2A-B subunits (Zhou et al., 2003; Junttila et al., 2007; Sablina et al., 2007). Therefore, loss of the RCN1 scaffold would result in the direct loss of PP2A holoenzymes that specifically require RCN1 for formation. Secondly, PP2A-A is a crucial component of the protein complex that transforms low-activity PP2A-C into a fully active enzyme (Hombauer et al., 2007). Based on single mutant analysis, RCN1 appears to be the preferred Arabidopsis scaffolding subunit for PP2A-C activation (Zhou et al., 2004). Therefore, the number of active PP2A-C subunits competent to form holoenzymes may be limiting in *rcn1* seedlings. This would create a scenario where bulk PP2A activity may be below a threshold level for regulation of cellular events. Thirdly, loss of RCN1 could result in coordinate degradation of PP2A-C. Previous immunoblot results did not reveal loss of PP2A-C (Zhou et al., 2004; Appendix 1). This suggests that PP2A-C accumulation is not affected by *rcn1,* although accumulation of PP2A-C has not been rigorously quantitated. Therefore *rcn1* mutant defects may arise through two general mechanisms, loss of specific

RCN1-dependent holoenzymes, and/or loss of holoenzymes due to decreased levels of active PP2A-C.

**Identifying PP2A Targets in the Ethylene Biosynthetic Pathway**

One of the best-characterized *rcn1* phenotypes is the short hypocotyl of dark-grown *rcn1* seedlings, which is caused by ethylene overproduction (Garbers et al., 1996; Larsen and Chang, 2001; Muday et al., 2006). In terms of identifying specific PP2A regulatory events in Arabidopsis, the *rcn1* ethylene overproduction phenotype was an attractive candidate for further analysis, since the ethylene biosynthetic pathway is short and has been clearly defined (Yang, 1984). Additionally, ethylene production is phospho-regulated, and protein kinases that regulate ethylene biosynthesis have been identified (Felix et al., 1994; Spanu et al., 1994; Tatsuki and Mori, 2001; Hernandez Sebastia et al., 2004; Liu and Zhang, 2004). However, at the time I began my Ph.D. thesis work, no specific protein phosphatases had been implicated in regulating ethylene biosynthesis.

Ethylene biosynthesis is largely controlled through modulating ACS enzyme activity, which occurs at multiple levels, introducing many possible points where PP2A could exert regulatory action. The most obvious candidates for PP2A regulation in Arabidopsis include the MAPK and CDPK target motifs in ACS isozymes, which play known and putative roles in modulating ethylene biosynthesis, respectively. Additionally phosphorylation within the conserved catalytic domain of ACS isozymes could regulate enzyme function. The ETO1

and EOL proteins regulate ethylene biosynthesis by controlling type 2 ACS isozyme turnover and are also putative phosphoproteins *in vivo.* The MAPK cascade that stabilizes type 1 ACS isozymes could also be regulated by dephosphorylation of constituent signaling components.

Our analysis of *rcn1* seedlings has shown that PP2A negatively regulates ethylene production through modulating the phosphorylation of type 1 ACS isozymes at MAPK target sites. Interestingly, PP2A may also regulate MAPK activity through an undefined mechanism. Type 2 ACS isozymes are positively regulated by PP2A activity, but the requirement for ETO1/EOLs and CDPK phosphorylation in this regulation is unclear. Importantly, the determinants (e.g. RCN1 versus PP2AA3) responsible for directing PP2A action on ethylene biosynthesis have also been refined by analysis of chimeric transgenic lines.

**PP2A Dephosphorylates Type 1 ACS Isozymes**

The functionality of the ACS isozyme family is pivotal in regulating total levels of ethylene biosynthesis. This, along with the importance of phosphorylation in modulating ACS enzyme stability, made ACS isozymes logical candidates for analysis as PP2A targets. Our analysis showed that ACS enzymatic activity was increased in *rcn1* mutants and that the ethylene-dependent inhibition of hypocotyl elongation observed in PP2A-limiting conditions required *ACS2* and *ACS6*, both of the catalytically active Arabidopsis type 1 ACS isozymes (Skottke et al., 2011). Plants lacking ETO1 overproduce ethylene due to increased type 2 ACS isozyme

protein stability (Woeste et al., 1999; Chae et al., 2003). The *eto1 rcn1* double mutant showed an additive hypocotyl elongation defect compared to either parental single mutant (Skottke et al., 2011). Together these data indicate that ethylene overproduction in *rcn1* plants occurs as a result of misregulation of type 1, and not type 2, ACS isozymes.

Additionally, ACS6 was hyperphosphorylated in *rcn1* and CT-treated wild-type seedlings. This indicates that the phosphorylation status of ACS6 is modulated by PP2A activity. Finally, biochemical analysis showed that PP2A interacts with ACS6 and that PP2A from wild-type but not *rcn1* seedling extracts is capable of dephosphorylating an ACS6 C-terminal peptide containing phosphorylated MAPK target sites (Skottke et al., 2011; Figure 1). This provides some of the most compelling biochemical evidence to date for PP2A-dependent dephosphorylation of a specific phospho-target in plants (Figure 1).

Although all Arabidopsis type 1 ACS isozymes are highly conserved, there are subtle differences between the MAPK phosphorylation sites in ACS6 versus ACS2 and ACS1 (see Chapter 1, Figure 9). The similar cantharidin sensitivity of *acs2* and *acs6* plants suggests that PP2A regulates both ACS6 and ACS2 (Skottke et al., 2011). Future experiments testing the ability of PP2A to interact with ACS1 and ACS2 and to dephosphorylate peptides corresponding to the C-termini of ACS1 and ACS2 will provide a more complete picture of PP2A regulation of type 1 ACS isozymes.

Although MAPK phosphorylation is sufficient to stabilize type 1 ACS isozymes in Arabidopsis (Liu and Zhang, 2004), both CDPK and MAPK phosphorylation are required for stabilization in tomato (Kamiyoshihara et al., 2010). This indicates that there are (at least some) mechanistic differences in stress-induced type 1 ACS isozyme stabilization between plant species. Treating tomato fruit with okadaic acid (500nM) increased ethylene synthesis (Kamiyoshihara et al., 2010). This concentration of okadaic acid is high enough to inhibit many PPP family protein phosphatases. However, in conjunction with our work on PP2A regulation of type 1 ACS isozymes in Arabidopsis, these okadaic acid data suggest that PP2A functions to constitutively oppose basal phosphorylation of type 1 ACS isozymes in tomato (Figure 1). Similar kinase- and phosphatase-dependent modulation of ACS enzyme activity has been observed in several plant species (Felix et al., 1994; Spanu et al., 1994), suggesting that PP2A regulation of ethylene biosynthesis is common in plants. It will be interesting to determine the extent of PP2A dephosphorylation of tomato type 1 ACS isozymes. For example, does PP2A only counteract MAPK phosphorylation or does PP2A dephosphorylate both CDPK and MAPK sites?

**Scaffold Function: Threshold Activity vs. Substrate Specificity**

Our data show that *rcn1* seedlings overproduce ethylene because PP2A fails to counteract basal MAPK-phosphorylation of type 1 ACS isozymes, increasing protein stability and driving ethylene synthesis (Skottke et al., 2011). Failure to

form PP2A holoenzymes competent to regulate type 1 ACS isozymes in *rcn1* seedlings could occur for several reasons. The most parsimonious explanation is that the PP2A holoenzyme that targets type 1 ACS isozymes contains a PP2A-B subunit that specifically requires the RCN1 scaffold for incorporation into a holoenzyme. In animals, some PP2A-B subunits show preferences for binding to either PP2A complexes containing PP2A-A$\alpha$ or PP2A-A$\beta$ scaffolds, providing a clear precedent for this idea (Zhou et al., 2003; Sablina et al., 2007).

Our analysis of the PP2A-A transgenic series demonstrated that *AYA* transgenic lines complement the short-hypocotyl phenotype of *rcn1* plants (Blakeslee et al., 2008). This suggests that PP2A complexes do not absolutely require RCN1 for proper targeting to type 1 ACS isozymes. If RCN1 is not directly necessary for PP2A-mediated dephosphorylation of type 1 ACS isozymes, an alternative hypothesis is that the PP2A holoenzymes present in *rcn1* plants are not capable of promoting threshold PP2A activity to support normal hypocotyl elongation. In this model, the abundance of AC core dimers (PP2AA2:C and PP2AA3:C) competent to bind regulatory PP2A-B subunits is limiting compared to the number of PP2A phosphosubstrates. This might create a situation where PP2A-B subunits must compete with each other for binding to AC core dimers. In this scenario, the PP2A-B subunit(s) responsible for targeting PP2A to type 1 ACS isozymes could be outcompeted by other PP2A-B subunits, either because of abundance or binding efficiency. Without knowing the identity of the PP2A-B subunit(s) responsible for targeting PP2A to type 1 ACS isozymes, it is difficult to

test this hypothesis. The AYA transgenic lines could be used to directly test the ability of PP2AA3 containing PP2A complexes to dephosphorylate ACS6 in the absence of RCN1. For this experiment, PP2A complexes would be immunoprecipitated from YFP-PP2AA3 plants using anti-YFP antibodies. The immunoprecipitated PP2A complexes would then be assayed for their ability to dephosphorylate a phospho-ACS6 C-terminal peptide. If the PP2A threshold model is correct, PP2AA3 PP2A complexes should dephosphorylate ACS6.

**Future Analysis of PP2A-Mediated Type 1 ACS Isozyme Regulation**

One of the most intriguing questions surrounding PP2A-dependent dephosphorylation of the ACS6 C-terminal MAPK target site is the identity of the regulatory PP2A-B subunit(s) involved in targeting PP2A to ACS6. With the help of Eliza Hamm, a very talented undergraduate, we have constructed a nearly comprehensive collection of homozygous *PP2A-B* T-DNA insertional lines, which will be useful in identifying the PP2A-B subunit(s) involved in PP2A-dependent regulation of ethylene biosynthesis (Hamm, Skottke, and DeLong, unpublished). Previous work has shown that some closely related PP2A-B subunits in all three Arabidopsis families (B, B', B") can be functionally redundant. For example, it was recently demonstrated that PP2A holoenzymes containing PP2A-$B^{Balpha}$ and PP2A-$B^{Bbeta}$ contribute to dephosphorylation-induced activation of nitrate reductase in Arabidopsis (Heidari et al., 2011). Additionally, several, but not all of the PP2A-B' family members are able to interact with and direct PP2A-mediated dephosphorylation of the brassinosteroid signaling component BZR1

(Tang et al., 2011). Finally, the Arabidopsis PP2A-$B^{B"}$ family members, B"$\alpha$ and B"$\beta$ both function in regulating 3-hydroxy-methylglutaryl coenzyme A reductase (HMGR) accumulation and activity (Leivar et al., 2011). For this reason, we are in the process of generating double mutants for all closely related *PP2A-B* family members.

Dark-grown *rcn1* mutants have a characteristic short-hypocotyl phenotype due to ethylene overproduction. Screening our pools of PP2A-B single and closely related PP2A-B double mutants for a short-hypocotyl phenotype similar to that of *rcn1* should allow us to identify the regulatory PP2A-B subunit(s) necessary for targeting type 1 ACS isozymes. It was recently reported that the double mutant *pp2ab'$\alpha$ pp2ab'$\beta$* displays a short-hypocotyl phenotype when grown in the dark (Tang et al., 2011). While a short-hypocotyl is often associated with ethylene production/signaling defects, the *pp2ab'$\alpha$ pp2ab'$\beta$* double mutant also has brassinosteroid signaling defects (Tang et al., 2011). Therefore PP2A-$B^{B'alpha}$ and PP2A-$B^{B'beta}$ are prime candidates for type 1 ACS regulation but it will be important to test the ethylene dependence of the *pp2ab'$\alpha$ pp2ab'$\beta$* short-hypocotyl phenotype.

We have observed ethylene overproduction in *rcn1* plants as a dark-specific phenomenon. It will be interesting to see if PP2A regulates type 1 ACS isozymes in light-grown tissues as well. Although adult *rcn1* plants do not show severe ethylene overproduction phenotypes, one more subtle observation is that *rcn1*

plants have thicker stems than wild-type plants (Larsen and Chang, 2001). It is unclear whether increased stem thickness in adult *rcn1* plants is due to local or tissue specific ethylene overproduction or enhanced ethylene responsiveness. In the future, type 1 ACS protein stability should be measured in wild-type and *rcn1* adult tissues, specifically stems. This will allow us to determine if PP2A-mediated regulation of type 1 ACS enzymes occurs throughout the plant life cycle or if it is restricted to the dark-growth developmental window. Knowledge of the PP2A-B subunits necessary for PP2A regulation of type 1 ACS isozymes will also inform our predictions about the scope of this regulation. For example, we expect PP2A regulation of type 1 ACS isozymes to occur in all tissues and developmental windows where the required PP2A-B subunits are expressed and type 1 ACS isozymes are present.

**PP2A as a Putative MAPK Phosphatase in Arabidopsis**

In addition to direct action on type 1 ACS isozymes, PP2A may regulate ethylene production and/or ethylene perception through regulation of MPK3. MPK3 and MPK6 function as part of a stress-responsive signaling cascade that positively regulates type 1 ACS isozyme stability (Liu and Zhang, 2004; Joo et al., 2008; Han et al., 2010). One report has suggested that MPK3 and MPK6 also function in ethylene signaling through phosphorylation-dependent modulation of EIN3 (an ethylene responsive transcription factor) protein stability (Joo et al., 2008). Analysis of hemi-double mutant *mpk3 mpk6/+* and *mpk6 mpk3/+* plants has also demonstrated that MPK3 and MPK6 are crucial regulators of plant development

(Hord et al., 2008; Wang et al., 2008). Our preliminary analysis of protein extracts from dark-grown seedlings suggests that MPK3 (but not MPK6) activity is increased in *rcn1* seedlings (Appendix 2; Figure 3). If these preliminary results are corroborated, it will be interesting to identify downstream targets affected by increased MPK3 activity in *rcn1* seedlings. This opens the possibility that increased phosphorylation and stability of type 1 ACS isozymes is the result of multi-level PP2A deregulation. Since *rcn1* protein extracts lose the ability to dephosphorylate a C-terminal ACS6 peptide, it is likely that the loss of PP2A-mediated type 1 ACS dephosphorylation is a major determinant of myc-ACS6 hyperphosphorylation and ethylene overproduction in *rcn1* seedlings. Additionally, if MPK3 activity is increased in *rcn1* seedlings, it is possible that myc-ACS6 hyperphosphorylation is also enhanced by increased MPK3 phosphorylation.

It would be possible to test the contribution of MPK3-dependent phosphorylation of type 1 ACS isozymes in *rcn1* seedlings through analysis of ethylene evolution in double mutant seedlings. It is well established that dark-grown *rcn1* seedlings produce more ethylene than wild-type seedlings as measured by headspace analysis of seedlings grown in airtight vials (Muday et al., 2006). If increased MPK3 activity contributes to type 1 ACS activity in *rcn1* seedlings, then the *rcn1 mpk3* double mutant should produce less ethylene than *rcn1* single mutants. Because RCN1 functions in ethylene signaling (as MPK3 also may do), growing the seedlings in the presence of silver nitrate to block ethylene perception should

allow for analysis of ethylene synthesis in the absence of confounding signaling effects.

The mammalian Raf (MAPK) signaling cascade culminates in the activation of Erk and is regulated by PP2A at several points (Figure 2). Raf signaling and regulation may be relevant to MPK3/6 activation because Erk is orthologous to MPK3/6 (Samuel et al., 2000). In the Raf signaling cascade, PP2A acts as a positive regulator of upstream signaling events by catalyzing activating dephosphorylation of the MAPK scaffold protein Ksr and activating dephosphorylation of Raf (Raabe and Rapp, 2003;Figure 2). PP2A also inhibits Raf signaling at the MAPK level by dephosphorylating Erk. This regulatory function may be conserved in plants because preliminary data suggests that MPK3 activity is increased in *rcn1* seedlings (Appendix 2). In mammals, Erk maintains homeostasis through negative feedback regulation (inactivating phosphorylation) of Raf and Mek kinases (reviewed in Ramos, 2008). Currently little is known about the regulation of MAPK signaling cascades in plants. This is highlighted by the fact that no MAPK scaffold proteins have been characterized in Arabidopsis (Wang, 2007). In the future it will be of interest to determine if PP2A regulates plant MAPK signaling at multiple levels like in the mammalian Raf signaling cascade. Additionally, whether MPK3/6 inactivate upstream kinases has yet to be addressed.

**Regulation of Type 2 ACS Isozymes by PP2A**

In addition to the PP2A-dependent dephosphorylation-induced destabilization of type 1 ACS isozymes, which is required to maintain low levels of ethylene production and proper hypocotyl elongation in the dark, we uncovered an unexpected role for PP2A as a positive regulator of type 2 ACS isozyme accumulation and stability (Skottke et al., 2011; Figure 3). Our biochemical analysis revealed that transgenic plants expressing myc-ACS5 (a representative type 2 ACS isozyme) had greatly reduced ACS5 accumulation in *rcn1* seedlings compared to wild-type. Additionally, genetic analysis of *ACS* contributions to hypocotyl elongation using *acs5* and *acs9* mutants suggests that ACS9 should behave similarly to ACS5 (Skottke et al., 2011). The phosphoregulation of type 2 ACS isozymes *in vivo* has not been clearly established. A peptide corresponding to the putative C-terminal ACS5 CDPK target site was phosphorylated *in vitro* by CDPK-activity-containing maize extracts, although at a lower rate than a peptide corresponding to the tomato LeACS2 CDPK phosphorylation target motif (Hernandez Sebastia et al., 2004). Therefore it is unclear if PP2A-dependent stabilization of ACS5 is mediated through the CDPK target motif or through an unrelated phosphorylation event. Interestingly, preliminary analysis of wild-type myc-ACS5 protein migration by SDS-PAGE and immunoblotting revealed two poorly separated bands, while myc-ACS5$^{eto2}$ migrated as a single species (Skottke and DeLong, unpublished data). This may indicate that ACS5 is phosphorylated *in vivo,* although further work such as phosphatase treatment is

needed to determine whether the myc-ACS5 doublet represents a difference in phosphorylation status.

Interestingly, the dramatic myc-ACS5 destabilization we observed in *rcn1* seedlings was dependent on the wild-type ACS5 C-terminus, as there were no differences in myc-ACS5$^{eto2}$ protein accumulation or stability between wild-type and *rcn1* plants (Skottke et al., 2011). Our preliminary co-immunoprecipitation experiments show that PP2A interacts with both myc-ACS5 and myc-ACS5$^{eto2}$ in wild-type plants (Appendix 2). Interaction between PP2A and myc-ACS5 suggests that PP2A may regulate the stability of type 2 ACS isozymes directly. However, since PP2A interacts with ACS5$^{eto2}$ but the *rcn1* mutation has no effect on ACS5$^{eto2}$ protein accumulation, it appears that the wild-type ACS5 C-terminus is required to translate PP2A binding into protein stabilization. This could indicate that PP2A acts on the intact CDPK phosphorylation site and/or that additional regulatory factors (like ETO1) are required for PP2A-mediated type 2 ACS isozyme stabilization.

If PP2A regulates ACS5 C-terminal phosphorylation, then binding to both ACS5 and ACS5$^{eto2}$ is not unexpected. In fact, there is a precedent for Arabidopsis PP2A binding to a protein motif that is separate/distinct from the site of dephosphorylation (Tang et al., 2011). If PP2A regulates ACS5 stability through CDPK site dephosphorylation, then ACS5 destabilization in *rcn1* seedlings suggests that CDPK phosphorylation is antagonistic to protein stability. A

peptide dephosphorylation assay could be used to test the ability of PP2A to dephosphorylate ACS5 at the CDPK site. A C-terminal ACS5 peptide could be phosphorylated with CDPK-containing extract (Hernandez Sebastia et al., 2004) with subsequent dephosphorylation assayed in protein extracts from wild-type and *rcn1* seedlings. If the phospho-ACS5 peptide is a good PP2A substrate then wild-type but not *rcn1* extracts would be expected to dephosphorylate the CDPK site.

The genetic interaction between *ETO1* and *RCN1* in regulating type 2 ACS isozyme stability could be tested using double mutant analysis. In this experiment, the protein half-life of myc-ACS5 would be assayed in wild-type, *eto1*, *rcn1*, and *eto1 rcn1* seedlings. The stability of myc-ACS5 in wild-type plants provides a benchmark for normal myc-ACS5 protein turnover. Myc-ACS5 should be stabilized and destabilized in *eto1* and *rcn1* seedlings, respectively (Wang et al., 2004; Skottke et al., 2011). If increased myc-ACS5 turnover in *rcn1* seedlings requires ETO1, then myc-ACS5 protein stability should be increased in *eto1 rcn1* seedlings compared to *rcn1* seedlings.

**Analysis of the PP2A-A Requirements for Proper PP2A Function**

The *rcn1* mutant, as a partial PP2A-loss-of-function line, has proven valuable in identifying processes regulated by PP2A. However, the very nature of the PP2A defect in *rcn1* plants is poorly understood. For example, RCN1, PP2AA2, and PP2AA3 proteins are all highly identical, but only *rcn1* plants have reduced PP2A

activity and pleiotropic phenotypes while *pp2aa2* and *pp2aa3* mutant plants appear phenotypically wild-type (Zhou et al., 2004). In order to better understand the generation of active PP2A complexes in Arabidopsis, we undertook an analysis of PP2A-A subunit specificity determinants, investigating the individual role *PP2A-A cis*-regulatory (promoter) sequence and *PP2A-A* coding sequence play in the formation of physiologically active PP2A complexes (Blakeslee et al., 2008). In order to dissect *PP2A-A* regulatory and coding sequence contributions to PP2A activity, *PP2A-A*$_{promoter}$*::YFP:PP2A-A* fusions were generated and transformed into *rcn1-1* mutant plants. We generated four fusion constructs designated *RYR – RCN1*$_{pro}$*::YFP-RCN1, RRY – RCN1*$_{pro}$*::RCN1-YFP*, *RYA – RCN1*$_{pro}$*::YFP-PP2AA3*, and *AYA – PP2AA3*$_{pro}$*::YFP-PP2AA3* (Blakeslee et al., 2008). Based on the strong *rcn1* phenotype and lack of an obvious *pp2aa3* phenotype, one might assume that the *PP2A-A* fusion constructs described above would be unequal in their ability to complement *rcn1* mutant phenotypes, behaving as an allelic series, where *RYR/RRY > RYA > AYA*. Therefore, the complete set of RYR, RYA, and AYA transformants will be referred to as the PP2A-A transgenic series. Transgene dosage was determined by Southern blotting and protein dosage was analyzed using immunoblotting and fluorimetric analysis (Blakeslee et al., 2008; Appendix 1). Physiologically relevant PP2A activity was determined across the PP2A-A transgenic series by assaying the ability of each construct to complement *rcn1-1* mutant phenotypes.

One surprising result of our *PP2A-A* subunit fusion-construct analysis was that *rcn1* defects displayed differential *PP2A-A* requirements for complementation. For example, the short-hypocotyl phenotype of dark-grown *rcn1* seedlings was fully rescued in single-copy *RYR, RYA,* and *AYA* transgenic plants. In contrast, *rcn1* defects in root tip patterning were only fully complemented in *RYR* (or RRY) transgenic lines (Blakeslee et al., 2008). This indicates that RCN1 is specifically necessary for proper regulation of root tip patterning but not hypocotyl elongation.

**PP2AA3 Fails to Promote PP2A Activity Above a Physiologically Relevant Threshold**

In our complementation analysis, the *RCN1* regulatory and coding regions were required for full rescue of root tip architecture. Although RCN1 is necessary, we do not fully understand why. One explanation is that RCN1 recruits a unique population of PP2A-B subunits. Alternatively, RCN1 may be required for maintaining PP2A activity above a threshold by ensuring the number of AC core dimers is not limiting. The best way to distinguish these possibilities is to identify the PP2A-B subunits required for proper root tip physiology and assay their binding affinities for RCN1, PP2AA2, and PP2AA3. This will address RCN1 requirements for holoenzyme formation. Additionally, model substrate dephosphorylation assays should give an approximation of how many active PP2A complexes are present in members of the PP2A-A transgenic series. To date, no one has undertaken a detailed biochemical analysis of bulk PP2A

activity in the PP2A-A transgenic series. Without activity data or the identity of the PP2A-B subunits required for root tip patterning it is difficult to discriminate between the direct RCN1-binding and AC core dimer-competition models for exclusion of the PP2A-B subunit(s) required for normal root growth.

Our analysis of hypocotyl elongation during inhibitor challenge suggests that PP2A activity is not fully restored in either *AYA* or *RYA* transgenic lines (Appendix 1). We found that when grown in ideal conditions, all members of the PP2A-A transgenic series were able to fully complement the short-hypocotyl phenotype of *rcn1* seedlings (Blakeslee et al., 2008). However, when challenged with the selective PP2A inhibitor cantharidin, single copy *RYA* and *AYA* lines were no longer able to support wild-type growth (Appendix 1). Multi-copy *RYA* and *AYA* transgenic plants showed a copy number-dependent increase in hypocotyl elongation rescue, suggesting that the number of functional PP2A AC core dimers in single-copy RYA and AYA transgenic plants may be limiting and that increasing transgene dosage rescues PP2A activity further. In order to address this more directly, the accumulation of YFP-PP2A-A should be quantified and compared to PP2A activity data for each member of the PP2A-A transgenic series. This will allow rigorous analysis of the relationship between PP2A-A subunit accumulation and PP2A activity.

Our analysis has shown that PP2A regulation of ethylene biosynthesis is complex. PP2A negatively regulates type 1 ACS isozymes through direct

dephosphorylation. PP2A-mediated inhibition of MPK3 may also provide additional negative regulation of type 1 ACS isozymes. Surprisingly, PP2A also positively regulates the accumulation of type 2 ACS isozymes. Major questions that remain include: What is the molecular mechanism underlying PP2A-dependent stabilization of type 2 ACS isozymes? What is the functional consequence of elevated MPK3 activity in *rcn1* seedlings? What is the identity of the PP2A-B subunits responsible for directing PP2A activity towards type 1 and type 2 ACS isozymes? And finally how is PP2A regulation of ethylene biosynthesis controlled in a developmental context?

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**FIGURES**

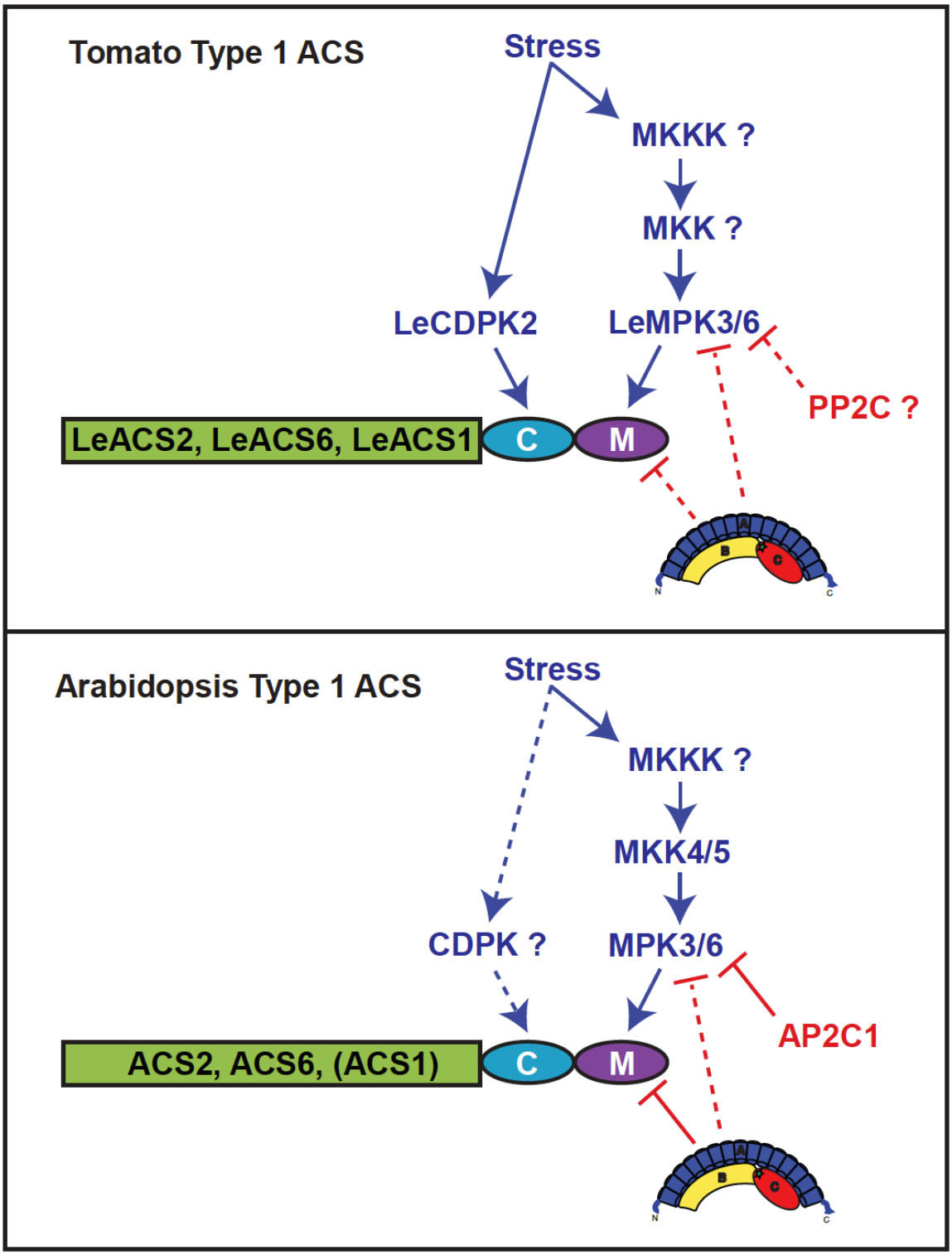
Tomato Type 1 ACS
Stress
MKKK ?
MKK ?
LeCDPK2
LeMPK3/6
PP2C ?
LeACS2, LeACS6, LeACS1
C
M
Arabidopsis Type 1 ACS
Stress
MKKK ?
MKK4/5
CDPK ?
MPK3/6
AP2C1
ACS2, ACS6, (ACS1)
C
M

**Figure 1. Posttranslational regulation of type 1 ACS isozyme stability in tomato and Arabidopsis.** Both tomato and Arabidopsis possess three type 1 ACS isozymes, ACS1, 2, and 6. In tomato stress cues such as wounding activate both LeCDPK2 and a MAPK cascade culminating in LeMPK3/6. The combined phosphorylation of CDPK and MAPK target sites within tomato type 1 ACS isozymes enhances protein stability (shown as blue arrows). In Arabidopsis, CDPK regulation of type 1 ACS isozymes is uncertain (dashed blue arrows) but stress does activate a MAPK cascade consisting of MKK4/5 and MPK3/6, which phosphorylate type 1 ACS C-termini. The PP2C, AP2C1 functions as a negative regulator of stress-induced ethylene biosynthesis by dephosphorylating and inactivating MPK3/6 (red bars). PP2A also negatively regulates ethylene biosynthesis by directly dephosphorylating type 1 ACS isozymes. Additionally, PP2A may negatively regulate MPK3, acting is a MAPK phosphatase similar to AP2C1. The roles of PP2A and PP2C in ethylene in tomato are still unclear (dashed red bars). However, inhibitor studies support a role for PP2A in dephosphorylating type 1 ACS isozymes similar to Arabidopsis.

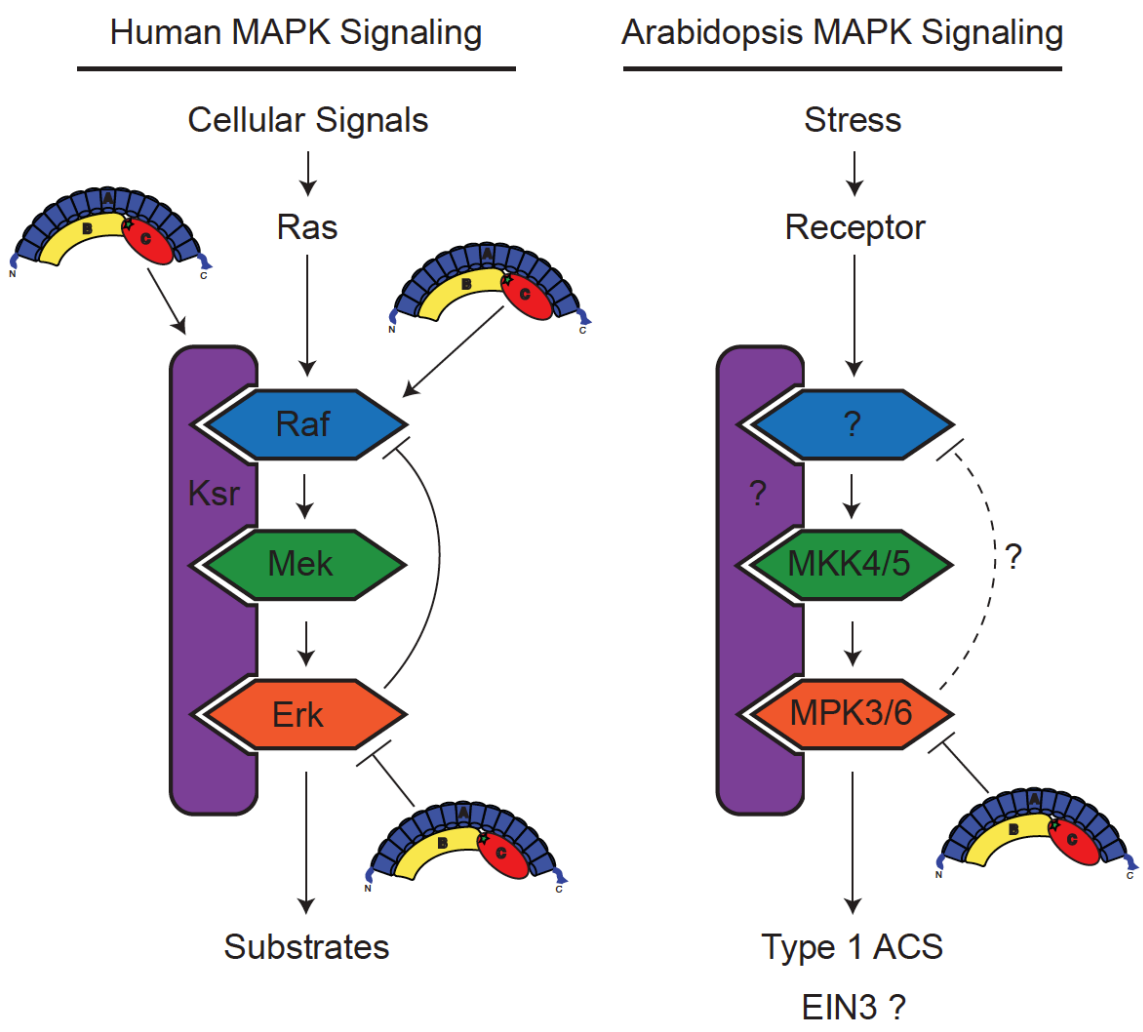


**Figure 2. PP2A-mediated regulation of MAPK activity in humans and Arabidopsis.** The Ras signaling cascade is a well-characterized MAPK signaling cascade regulated by PP2A and with homology to MPK3/6 in Arabidopsis. In humans, Ras stimulation leads to the activating PP2A-mediated dephosphorylation of Raf (a MEKK) and Ksr (a MAPK scaffold). These dephosphorylation events promote the assembly of the Ksr-Raf-Mek-Erk signaling complex. Interestingly, PP2A also represses Erk signaling by dephosphorylating Erk directly. Activated Erk also provides negative feedback by phosphorylating Raf. In Arabidopsis, a MAPK cascade consisting of MKK4/5 and MPK3/6 mediate stress-induced increased ethylene production by phosphorylating type 1 ACS isozymes. The identity of the upstream MKKK and the involvement of a MAPK scaffold in this cascade are unknown. Our preliminary data suggests that PP2A negatively regulates MPK3, similar to the PP2A-Erk paradigm. The role of MPK3/6 feedback regulation of upstream kinases and the involvement of upstream PP2A regulation in Arabidopsis are unknown.

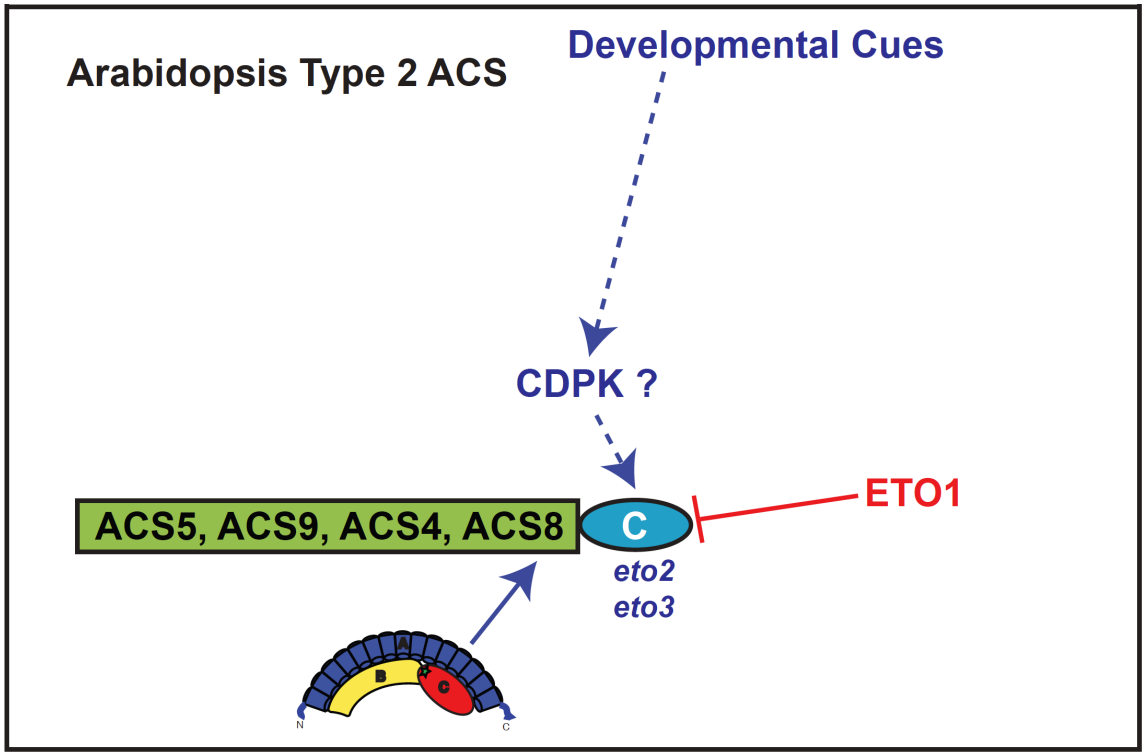


**Figure 3. Posttranslational regulation of Arabidopsis type 2 ACS isozyme stability.** In Arabidopsis developmental cues lead to the stabilization of type 2 ACS isozymes, although it is unclear if stabilization is mediated through CDPK phosphorylation. ETO1 is an E3-ubiquitin ligase substrate specificity adaptor protein that specifically recognizes the C-terminus of type 2 ACS isozymes and targets them for proteasome-dependent turnover. The *eto2* and *eto3* mutations alter the ETO1 binding motif in ACS5 and ACS9, respectively, which leads to increased protein stability and ethylene production. PP2A promotes the accumulation of type 2 ACS isozymes (blue arrow). PP2A interacts with both ACS5 and ACS5$^{eto2}$, suggesting that the site of interaction is located outside of the ETO1 binding motif.

# APPENDIX 1: REGULATION OF ARABIDOPSIS PP2A ACTIVITY *IN VIVO*

Unpublished data

I performed all of the experiments presented in this appendix.

## INTRODUCTION

PP2A is an extremely abundant serine/threonine protein phosphatase with regulatory roles in many major cellular processes (Janssens and Goris, 2001). For this reason PP2A activity is highly regulated. Regulation occurs at multiple levels including transcription and translation of individual PP2A subunits, as well as control of holoenzyme assembly and catalytic activation of PP2A-C. PP2A-A and PP2A-B subunits impart substrate specificity and distinct subcellular localization patterns when complexed with PP2A-C subunits in heterotrimeric complexes *in vivo* (Janssens and Goris, 2001). The binding of PP2A-A and PP2A-B subunits also limit indiscriminate dephosphorylation of non-substrate phosphoproteins (Price and Mumby, 2000). Uncontrolled dephosphorylation by PP2A-C in the absence of regulatory PP2A-A and PP2A-B subunits would constitute a dire risk to cellular phospho-homeostasis.

The need for extremely fine-tuned regulation of PP2A activity is highlighted by the fact that PP2A accounts for up to 1% of the total cellular proteome (Ruediger et al., 1991; Lin et al., 1998). In fact, several mammalian viruses with cellular transformation capabilities achieve tumorigenesis though targeted misregulation of PP2A. SV40 and polyoma virus achieve cellular transformation (in part) by using their small-T antigens to displace regulatory PP2A-B subunits from heterotrimeric complexes, subverting normal PP2A function (reviewed in Mumby, 2007). Additionally, point mutations in the mammalian A$\alpha$ and A$\beta$ PP2A

scaffolding subunits which abrogate binding to specific regulatory PP2A-B subunits also result in aberrant PP2A function and tumorigenesis (Zhou et al., 2003; Mumby, 2007; Sablina et al., 2007). Clearly, fine-tuned regulation of PP2A activity is a prerequisite to normal cellular biology. In accordance with this, it is logical that a mechanism for regulating the catalytic activity of nascent PP2A-C should exist.

A possible mechanism for controlling nascent PP2A-C activity until regulatory proteins (A + B) are bound has been uncovered in *Saccharomyces cerevisiae*, where maturation of the PP2A catalytic subunit appears to be coupled to holoenzyme assembly (Hombauer et al., 2007). Catalytic activity of PP2A-C is dependent on the proper coordination of two $Mn^{2+}$ ions (Xing et al., 2006; Xu et al., 2006). Nascent PP2A-C is folded in a low-activity conformation and lacks the coordinated $Mn^{2+}$. PP2A-C remains in a low-activity conformation until the PP2A phosphatase activator, PTPA alters the structure of the PP2A-C catalytic center, allowing for full activity (Fellner et al., 2003; Chao et al., 2006; Leulliot et al., 2006).

In order to activate PP2A-C, PTPA interacts with a complex containing PP2A-A and PP2A-C (Hombauer et al., 2007; Figure 1). Yeast strains lacking PTPA have a severely retarded growth phenotype resulting from an approximate 80% decrease in PP2A activity (Fellner et al., 2003). Additionally, PP2A-A deletion strains show similar defects in growth and PP2A activity (Hombauer et al., 2007).

These data indicate that the PP2A-C subunit requires both PP2A-A and PTPA to gain full activity.

Upon activation by PTPA, the AC core dimer can be methylated by PPM1. Yeast strains deleted for *PPM1* have an approximate 60% drop in PP2A activity, suggesting that upon activation by PTPA, the AC core dimer may require methylation by PPM1 to progress with holoenzyme assembly (Fellner et al., 2003; Hombauer et al., 2007). However, since only a subset of PP2A-B subunits absolutely require PP2A-C C-terminal Leu309 methylation for incorporation into a holoenzyme complex, the necessity of methylation by PPM1 for progression of holoenzyme assembly remains uncertain (Janssens et al., 2008). It is possible that binding of PPM1 to the AC core dimer (rather than catalysis of methylation *per se*) functions as a signal for recruitment of PP2A-B subunits. The function of PPM1/LCMT in Arabidopsis is poorly understood.

PME1 is known to stably associate with an inactive pool of PP2A-C subunit (Ogris et al., 1999). PME1 opposes the action of PTPA by displacing the $Mn^{2+}$ ions within PP2A-C (Xing et al., 2008). Co-immunoprecipitation experiments have shown that PP2A-A interacts with PME1–PP2A-C in the inactive complex (Ogris et al., 1999; Figure 1). Thus, PP2A-A interacts with PP2A-C in both the inactivating complex containing PME1 as well as the activating complex containing PTPA. Therefore, PP2A-A is ideally poised to function in the still

poorly understood mechanism that senses PP2A activity levels and acts as a cellular PP2A biogenesis checkpoint.

If this were true, loss of PME1 might interfere with the ability of PP2A-A to properly recruit PTPA, interfering with PP2A biogenesis. In line with this prediction, *pme1Δ* yeast strains show a modest (~30%) drop in PP2A activity (Hombauer et al., 2007). Interestingly, overexpression of PME1 results in an approximate 70% decrease in PP2A activity, which is similar to the decrease in PP2A activity observed in the yeast strains lacking either PP2A-A or PTPA (Hombauer et al., 2007). Interestingly, human PME1 contains a functional nuclear localization motif (NLS) and normally associates with inactive PP2A-C within the nucleus (Longin et al., 2008). Overexpression of either wild-type PME1 or PME1 lacking the NLS failed to alter PP2A activity in human cell culture experiments (Longin et al., 2008). These data suggest that PME1 surveillance of inactive PP2A-C is complicated and may vary from species to species. The role of PME1 in Arabidopsis is unknown.

The yeast holoenzyme biogenesis model explains the PP2A activity defect in strains lacking PP2A-A, since PP2A-C would be unable to interact properly with PTPA and therefore remain crippled as a phosphatase. This model could explain the PP2A defect of *rcn1* Arabidopsis plants if RCN1 is the preferred scaffold for the PP2A-C activation complex. If PTPA interacts with RCN1 more efficiently than with PP2AA2 or PP2AA3, then failure to activate PP2A-C would account for

the PP2A defect observed in *rcn1* but not *pp2aa2* or *pp2aa3* plants (Zhou et al., 2004). In order to probe the role of PP2A-C methylation/demethylation in normal PP2A function in Arabidopsis, I obtained *lcmt* and *pme1* mutants and analyzed their effect on PP2A function *in vivo*.

In other model systems, PTPA, LCMT, and PME1 are well-established PP2A regulatory factors whose function modulates PP2A activity. In addition, our previous analysis of single and multiple Arabidopsis PP2A-A loss-of-function mutants clearly establish the PP2A-A scaffold as a positive regulator of PP2A activity (Deruere et al., 1999; Zhou et al., 2004; Blakeslee et al., 2008). The short-hypocotyl phenotype observed in dark-grown *rcn1* seedlings is due to decreased PP2A activity and can be recapitulated by treating wild-type seedlings with a PP2A inhibitor (e.g. cantharidin; Deruere et al., 1999; Blakeslee et al., 2008). We previously described the ability of transgene combinations consisting of either the *RCN1* or *PP2AA3* promoter driving expression of *YFP* fused to the coding sequence of either *RCN1* or *PP2AA3* to complement the *rcn1* short-hypocotyl phenotype when transformed into *rcn1-1* seedlings (Blakeslee et al., 2008). Transgenic lines are named based on a three-letter abbreviation (using R for RCN1, A for PP2AA3, and Y for YFP) designating the promoter source, the YFP tag, and the PP2A-A coding sequence. For example, the *RCN1* promoter driving an *YFP-RCN1* fusion is designated *RYR*. *RYR, RYA,* and *AYA* constructs were transformed into *rcn1-1* mutant plants, and lines harboring from 1 to >10 copies of the transgene were propagated.

Interestingly, while complementation of some *rcn1-1* phenotypes required RCN1 expression (e.g. root architecture), any additional *PP2A-A* dosage was able to rescue dark-grown hypocotyl elongation (Blakeslee et al., 2008). A detailed analysis of root architecture defects observed across the PP2A-A transgenic series indicated that transgenic *RYR* lines were able to completely complement *rcn1* defects while *RYA* lines showed moderate defects and *AYA* lines showed the lowest degree of complementation (Blakeslee et al., 2008). This suggests that *RYR, RYA,* and *AYA* transgenes may promote distinct levels of PP2A activity.

The stoichiometry of PP2A subunits in Arabidopsis is not yet defined and may vary in roots versus shoots. It is possible that the accumulation of PP2A-A is limiting for PP2A activity. We wanted to quantify PP2A-A subunit accumulation and to confirm that increasing *YFP-PP2A-A* gene dosage leads to increases in YFP-PP2A-A subunit accumulation. The chemiluminescent detection used in our previous analysis of PP2A-A accumulation in transgenic lines (Blakeslee et al., 2008) prevents rigorous quantitation of immunoblotting results. Therefore, we tested a fluorospectrometric approach to quantitate YFP levels. Low expression levels and the presence of autofluorescent molecules in plant extracts limit the use of this technique (reviewed in Johansen et al., 2007). However, because we are specifically interested in etiolated hypocotyls and because PP2A-A is ubiquitously and abundantly expressed, we were able to use this system to

provide for rapid, inexpensive and highly quantitative assessment of transgene expression levels. In order to understand the contributions that RCN1 and PP2AA3 make to promoting and regulating PP2A activity, I further analyzed the effect of PP2A-A subunit dosage on PP2A function.

## RESULTS

### Arabidopsis PME1 lacks a nuclear localization sequence (NLS)

We and others (Wu et al., 2011) identify At1g02100 as a putative LCMT/PPM1. In additon, BLAST identifies a single gene At4g10050 as PME1. Human and Arabidopsis PME1 share 85% sequence similarity but interestingly, the human PME1 contains a functional NLS, which is lacking in both Arabidopsis and yeast (Figure 2).

### *lcmt1-1* seedlings exhibit hypocotyl elongation defects

In order to test the hypothesis that *LCMT* (At1g02100) functions in PP2A biogenesis in Arabidopsis*,* a homozygous T-DNA insertion mutant in *LCMT* was ordered from the Arabidopsis Biological Resource Center and designated *lcmt-1* (SALK_079466.55.40; Alonso et al., 2003). The T-DNA insertion in *lcmt-1* is predicted to disrupt the fourth of 11-12 alternatively spliced exons. Additionally, a homozygous T-DNA insertion mutant in *PME1* (At4g10050) was obtained and designated *pme1-1* (SALK_079539C). The *pme1-1* lesion is predicted to disrupt the first of 13 exons. These mutants were assayed for effects on hypocotyl elongation and PP2A-C subunit accumulation.

Since ethylene is a potent inhibitor of dark-grown hypocotyl elongation and since PP2A function is antagonistic to overall ethylene production in dark-grown seedlings, we can use hypocotyl length as readout for PP2A activity (Skottke et al., 2011). Additionally, seedlings treated with the selective PP2A inhibitor

cantharidin show dose-dependent decreases in hypocotyl elongation, confirming that phosphatase activity correlates with hypocotyl elongation (Deruere et al., 1999). In hypocotyl elongation assays, *lcmt-1* mutant seedlings exhibit a growth defect similar to that of *rcn1* seedlings (Figure 3). This suggests that *lcmt* and *rcn1* mutant seedlings have similar reductions in PP2A activity. Interestingly, *pme1-1* mutant seedlings do not exhibit obvious hypocotyl defects and are not statistically different from wild-type seedlings in our elongation assay (Figure 3).

Because of the decrease in overall PP2A activity caused by loss of RCN1 function, *rcn1* seedlings exhibit increased sensitivity to CT in several assays (Deruere et al., 1999). To determine whether this is also true for *lcmt-1,* we assayed for inhibition of *lcmt-1* hypocotyl elongation in the presence of CT (Figure 2). *lcmt-1* seedlings show a modest increase in cantharidin sensitivity in our hypocotyl elongation assay (Figure 3).

**PP2A-C subunit accumulation is increased in *lcmt-1* seedlings**

Since methylation/demethylation of PP2A-C has been implicated in PP2A holoenzyme assembly, we assayed the accumulation of both PP2A-A and PP2A-C subunits in wild-type (Col), *rcn1-6, lcmt-1,* and *pme1-1* seedlings using immunoblotting with antibodies specific to PP2A-A and PP2A-C. Accumulation of scaffolding PP2A-A subunits was very similar between wild-type, *lcmt-1* and *pme1-1* plants; as expected, *rcn1* plants exhibited normal PP2AA2 and PP2AA3 accumulation (Zhou et al., 2004; Figure 4). This suggests that there is no

aberrant PP2A-A protein accumulation in *lcmt* or *pme1* seedlings. Surprisingly, PP2A-C subunit accumulation is drastically increased in *lcmt-1* seedlings compared to either wild-type or *rcn1* (Figure 4). Like many anti-PP2A-C antibodies, the anti-PP2A-C antiserum used in this immunoblot was raised against the PP2A-C carboxy-terminus and preferentially recognizes unmethylated PP2A-C (Ogris et al., 1999; our unpublished data). For this reason, the PVDF immunoblotting membrane was treated with NaOH prior to blocking and immunoblotting (see Methods) to remove ester-modifications, including PP2A-C Leu309 methylation. Given this treatment, the PP2A-C signal in each lane should represent the total (originally methylated and unmethylated) PP2A-C subunit present in these extracts. This result suggests that loss of LCMT function alters regulation of PP2A-C accumulation in Arabidopsis.

**PP2A-A dosage modulates PP2A activity**

In order to probe the robustness of hypocotyl elongation complementation in the PP2A-A transgenic series, seedlings were grown in the presence of the selective PP2A inhibitor cantharidin (CT). Although all transgenic lines (*RYR, RYA*, and *AYA*; single- and multi-copy transformants) were able to complement hypocotyl elongation under optimal growth conditions (Blakeslee et al., 2008; Figure 5A), multiple copies of RYA and AYA fusions were required to fully complement in the presence of CT. Only *RYR* transgenic lines were able to fully complement hypocotyl elongation when present in a single-copy (Figure 5B). Two copies of the *RYA* transgene (*RYA 32-3*) were sufficient to allow wild-type hypocotyl

elongation when grown on CT but a single copy (*RYA 28-4*) was not. Even up to 3 inserts of the *AYA* transgene (*AYA 2-3, AYA 6-1,* and *AYA 16-5*) were not sufficient to promote wild-type growth on CT, but 4 were (*AYA 100-4*), indicating that although *AYA* lines complement in optimal growth conditions, the complementation is not robust.

**Using fluorescence to measure YFP-A subunit accumulation**

The data described above suggest that PP2A-A subunit accumulation is limiting for PP2A activity in the *rcn1* background. This is especially interesting because PP2A activity levels are very tightly regulated and increasing PP2A-C subunit gene dosage and mRNA levels has no effect on PP2A activity (Baharians and Schonthal, 1998; Lizotte et al., 2008). Since *PP2A-A* transgene copy number correlates with the strength of rescue for lines expressing YFP-PP2AA3, we wanted to develop a method to allow quantification of YFP-PP2A-A expression in each of the transgenic lines.

Using YFP fluorescence as readout for PP2A-A subunit accumulation, the ability to complement hypocotyl elongation under PP2A-challenging conditions appears to correlate with expression of the PP2A-A subunit (Figure 5B, C). Additionally, comparing PP2A-A accumulation in the multi-copy line *RYR P1H9* with the single copy *RYR 85-1* and with the accumulation for double-copy *RYA 32-3* and single-copy *RYA 28-4*, confirms that PP2A-A accumulation does not increase linearly with transgene copy number (Figure 5C). *RYR 85-1* carries one insert and *RYR*

*P1H9* carries greater than 10 inserts yet *RYR P1H9* seedlings do not accumulate ten times more PP2A-A than *RYR 85-1*. Because our initial segregation and hybridization screens eliminated multi-insert and high-copy number transformants from further analysis, most lines in our collection carry one to three transgene copies. PP2A-A accumulation in *RYR P1H9* suggests that there may be an upper limit of PP2A-A accumulation, however, work with lines specifically designed to drive extremely high levels of PP2A-A will better address this question.

## DISCUSSION

The *rcn1*-like phenotype of dark-grown *lcmt-1* seedlings suggests PP2A deficiency and ethylene overproduction. Treatment with AVG (an inhibitor of ethylene biosynthesis) restores hypocotyl elongation in *lcmt-1* seedlings (Booker and DeLong, unpublished). This supports the idea that reduced hypocotyl elongation in *lcmt* seedlings is a result of ethylene overproduction. Given the role of LCMT in regulating PP2A activity in other organisms, it is likely that *lcmt* defects in Arabidopsis result from altered PP2A activity. Based on our previous work, one hypothesis accounting for the *lcmt-1* short-hypocotyl phenotype is that *lcmt-1* seedlings fail to properly dephosphorylate type 1 ACS isozymes. This would lead to increased ethylene production and reduced hypocotyl elongation similar to the *rcn1* mutant (Skottke et al., 2011). Our hypocotyl elongation assays in the presence and absence of a PP2A inhibitor challenge suggest that as in yeast, disruption of LCMT/PPM1 in Arabidopsis results in decreased overall PP2A activity.

Sequence comparison shows that Arabidopsis and yeast PME1 lack a NLS. Nuclear localized PME1 would be poorly situated to function as a negative regulator of PP2A-C activity prior to PP2A holoenzyme assembly since nascent PP2A-C will likely be cytoplasmic. This may indicate that fungal (and by analogy plant) PME1 surveys an inactive a pool of PP2A-C which is only (re)activated by PTPA upon cellular need, while human PME1 performs a more specialized

nuclear task. This may account for the observed discrepancies between human and yeast PME1 experiments.

One alternative explanation is that the cancerous human cell lines (COS and HeLA) used for human PME1 localization and overexpression studies do not provide an accurate account of PME1 function in normal human cells. Two lines of evidence support this notion. First, cancerous cell lines often display abnormal PP2A regulation, which may compromise analysis of normal PP2A function in these lines (Mumby, 2007). Second is that analysis of a mouse PME1 knockout revealed a drastic drop (~4-fold) in PP2A activity and a post-natal lethality phenotype (Ortega-Martinez et al., 2007). This indicates that PME1 is a positive regulator of PP2A activity in mice (and presumably other mammals as well).

Surprisingly, *pme1-1* mutant plants did not display an obvious phenotype in our assays. In both yeast and humans, PME1 function is required for full PP2A activity. Yeast lacking PME1 display growth defects and decreased PP2A activity (Hombauer et al., 2007). One possible explanation for the lack of an obvious phenotype in *pme1-1* seedlings is that the T-DNA insert does not fully disrupt gene function. Although this T-DNA line represents the best available insertional disruption of *PME1* (occurring in an early exon), it is possible that PME1 function is retained. Further analysis of the *pme1-1* allele is required to determine whether it impairs PME1 function.

The accumulation of functional PP2A is highly regulated, which has made overexpression of PP2A very difficult (Baharians and Schonthal, 1998; Lizotte et al., 2008). Therefore it is surprising that PP2A-A subunit accumulation and physiologically evident PP2A activity (i.e. regulation of hypocotyl elongation) show a certain degree of flexibility. It appears that PP2A-A expression is allowed within certain limits and that within these limits, variation in PP2A-A expression is able to alter PP2A activity. The low-end of the allowed PP2A-A accumulation spectrum is defined by the PP2A-A double mutants *rcn1 pp2aa2* and *rcn1 pp2aa3*. These plants display severe growth defects that are associated with severely decreased PP2A activity (Zhou et al., 2004). The upper limit of the PP2A-A accumulation spectrum is defined by multi-copy transformants like *RYR P1H9,* which carries more than 10 insertions of *YFP-RCN1* but does not accumulate much more protein than a single-copy insert, *RYR 74-11* (Blakeslee et al., 2008; Figure 5). More careful analysis of PP2A activity in *RYR P1H9* plants will be necessary to determine if additional RCN1 dosage can elevate PP2A activity beyond wild-type levels. Within the *AYA* transformant series, ranging from the multi-copy *AYA 100-4* to the single copy *AYA 2-3*, accumulation and PP2A activity also correlate (Figure 5B, C).

There appear to be significant contributions to PP2A function from both the PP2A-A promoter sequence and coding region, consistent with previous analysis (Blakeslee et al., 2008). This is evident when comparing the degree of hypocotyl elongation rescue between RYA and AYA lines (Figure 5). Transgenic lines

expressing PP2AA3 driven by the *RCN1* promoter rescue hypocotyl elongation when challenged by CT better than transgenic lines harboring the same number of insertions expressing PP2AA3 driven by the *PP2AA3* promoter. Therefore, although both the *RCN1* and *PP2AA3* promoters drive ubiquitous and high levels of expression, the *RCN1* promoter provides a stronger contribution to PP2A activity than the *PP2AA3* promoter when driving expression of the same PP2A-A subunit. PP2A-A amino acid sequence also impacts the strength of rescue since *RYR* single-copy transformants (74-11) rescue better than single-copy *RYA* transformants (28-4) when challenged with CT.

We propose a model where physiologically relevant PP2A activity (as determined by proper hypocotyl elongation) in Arabidopsis is modulated by LMCT and PP2A-A subunit levels based on the analysis of *lcmt-1* mutant seedlings as well as the transgenic PP2A-A dosage series presented here (Figure 6). Based on our hypocotyl elongation assays, LCMT function appears necessary for generation of ethylene-regulating PP2A activity (Figure 2). Additionally, PP2A-A subunit dosage appears to modulate PP2A activity, but only within an allowed window. Transgenic plants harboring many copies of *YFP-PP2A-A* (see *RYR P1H9* in Figure 5B, C) do not show linear increases in PP2A-A accumulation and PP2A activity. In contrast, reduced PP2A-A accumulation appears detrimental to PP2A activity (see AYA series in Figure 5B, C). Many questions still remain and more work is needed to identify the key regulatory mechanisms governing PP2A regulation in Arabidopsis.

## MATERIALS AND METHODS

### Plant material

*PME1* (At4g10050) encodes 13 exons. The *pme1-1* (SALK_079539C) allele contains a T-DNA insertion in exon 1. *PPM1* (At1g02100) encodes 12 exons. The *ppm1-1* (SALK_079466.55.40) allele contains a T-DNA insertion the fourth exon

### Hypocotyl elongation assays

Seedlings for each genotype were surface sterilized in a 20% bleach, 0.1% SDS solution for 10 minutes, washed three times, and stratified in 0.1% agar for 3 days at 4°. Seedlings were sown on media containing either 0 or 3 μM cantharidin, light-treated for 16 hours, and then placed vertically in the dark for 5 days. After 5 days of growth in the dark, plates were scanned and hypocotyl lengths were measured using Image J as in (Skottke et al., 2011).

### Immunoblotting

Wild-type, *rcn1*, *lcmt-1,* and *pme1-1* seedlings were grown in the dark for 5 days, frozen and ground in liquid nitrogen, and immediately boiled in 4X loading buffer. Extracts were centrifuged at 13K rpm for 15 minutes at 4° and the supernatant (extract) was transferred to a new tube. Extracts were separated by SDS-PAGE on 10% acrylamide gels and transferred to a PVDF membrane. After transfer,

the PVDF membrane was treated with 0.2 N NaOH for 20 minutes at 30°C to remove all C subunit methylation. After base treatment, membranes were neutralized, then blocked in 5% w/v non-fat dry milk and probed with anti-RCN1 antibodies to detect all three PP2A-A subunits and anti-C antibodies to detect total PP2A-C subunit accumulation.

**YFP-PP2A-A fluorescence assay**

Transgenic and control seedlings were grown on MS1 plates in the dark for 5 days and then frozen in liquid nitrogen. Protein extracts were made by grinding seedlings to a fine powder in liquid nitrogen, then suspending ground tissue in 100mM HEPES, pH 7.5, 5mM EDTA, 5mM EGTA, 10% glycerol, 1mM PMSF, 2mM benzamidine, and 1µg/ml aprotinin and leupeptin on ice. Extracts were centrifuged at 13K rpm for 15 minutes at 4°C and the supernatant was transferred to a new tube on ice. Extracts were then diluted to a concentration of 1µg/µl and 100µl of extract was added per well to a 96-well plate. Fluorescence was measured in quadruplicate on a Fluoroskan Ascent microplate reader.

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**FIGURES**

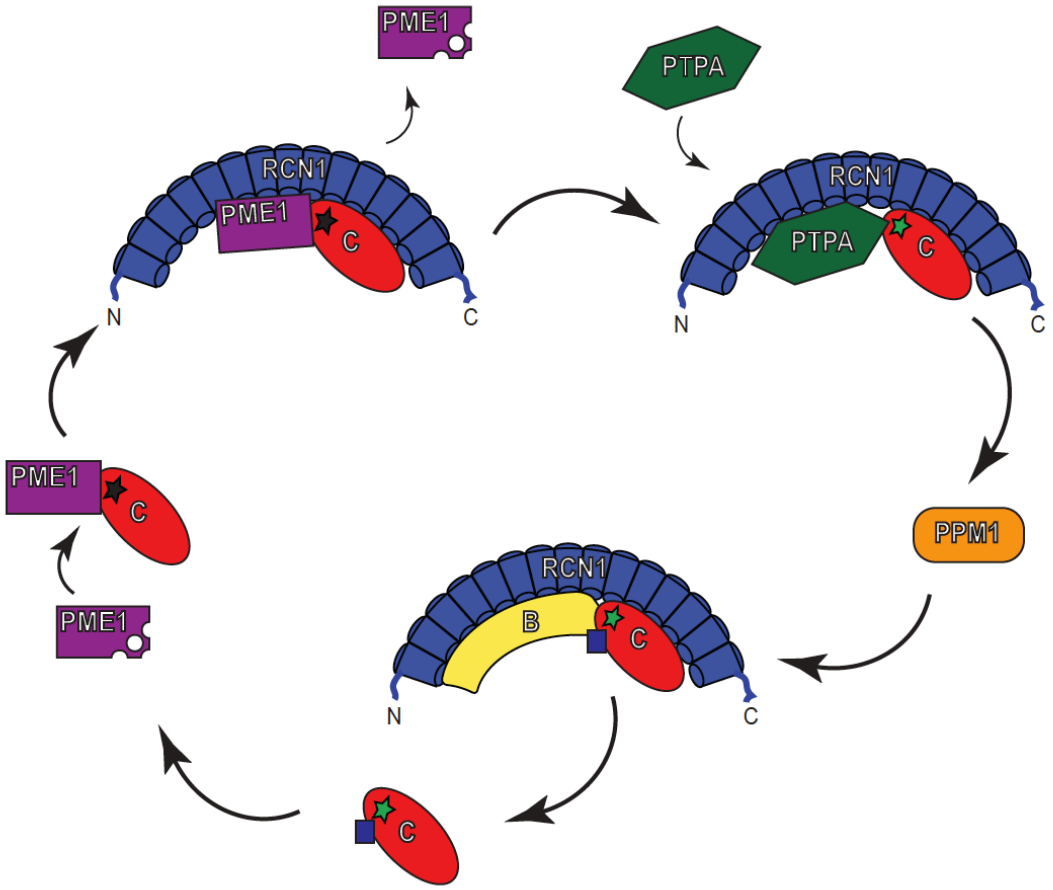



**Figure 1. Generation of active PP2A-C is coupled to PP2A holoenzyme assembly.** In this model based on work in *Saccharomyces cerevisiae*, nascent, low-activity PP2A-C is depicted with a black star in the active site. Low-activity PP2A-C is bound and kept inactive by PME1. PME1 only gains full catalytic activity (depicted as a completely filled-in box) upon binding to PP2A-C (Xing et al., 2008). The PME1-PP2A-C complex interacts with PP2A-A (Ogris et al., 1999). Upon passing the still poorly understood cellular PP2A quality control mechanism, PME1 releases from the AC core dimer and PTPA binds, forming a PTPA-PP2A-A-PP2A-C complex (Hombauer et al., 2007). At this point, PP2A-C is acted on by PTPA, which transforms the catalytic subunit into an active structure with the ability to properly coordinate two $Mn^{2+}$ ions in the catalytic center. After activation, PTPA is released from the AC core dimer, which is then free to be PP2A-C Leu309 methylated (blue square) by PPM1. After methylation / PPM1 association, regulatory B subunits join the AC core dimer and form functional heterotrimeric PP2A holoenzymes.

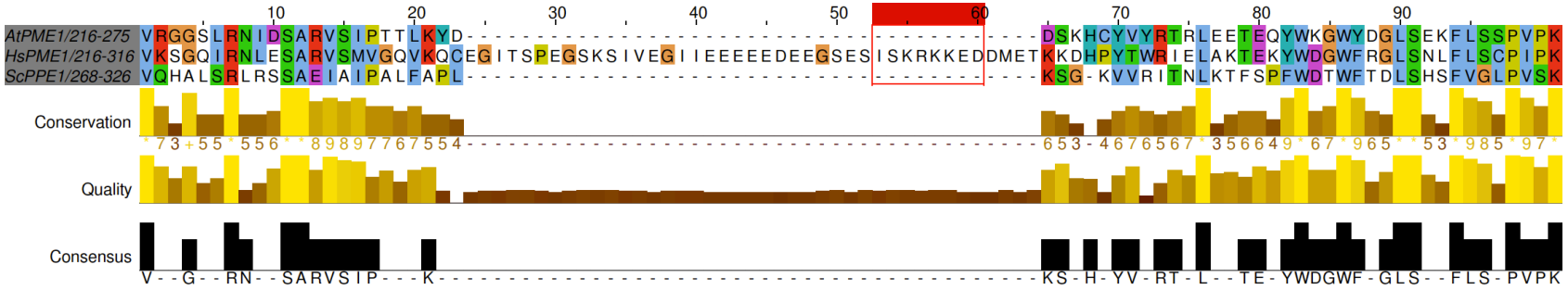

10
20
30
40
50
60
70
80
90
AtPME1/216-275
HsPME1/216-316
ScPPE1/268-326
Conservation
Quality
Consensus

**Figure 2. Arabidopsis PME1 lacks the NLS found in HsPME1.** A partial sequence alignment of AtPME1, HsPME1, andScPPE1 shows that the middle domain of HsPME1 that contains the nuclear localization motif ISKRKKED (highlighted in red) is not found in either AtPME1 or ScPPE1.

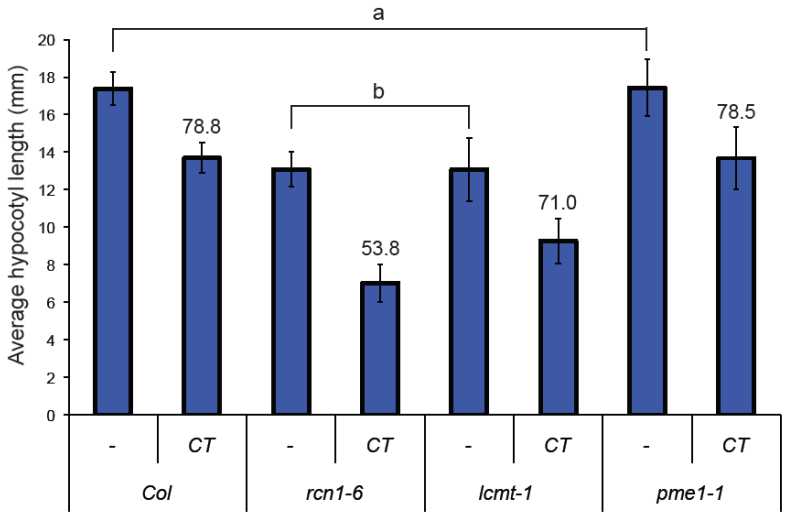



**Figure 3. *lcmt-1* seedlings have a dark-grown hypocotyl defect similar to *rcn1-6*.** After 5 days of growth in the dark on media containing either 0 or 3μM cantharidin (CT), the average hypocotyl lengths of wild-type (Col), *rcn1-6*, *lcmt-1*, and *pme1-1* seedlings were assessed. On media lacking CT, Col and *pme1-1* seedlings display no statistical difference in hypocotyl elongation (a). *rcn1-6* and *lcmt-1* seedlings show no difference in hypocotyl elongation from each other, but are significantly shorter than wild-type ($p < 8.0 \times 10^{-43}$; b). Interestingly, both *rcn1-6* and *lcmt-1* also show increased response to CT compared to wild-type or *pme1-1* seedlings. Percentage values shown represent length relative to untreated seedlings of that genotype; error bars show standard deviation (n > 30)

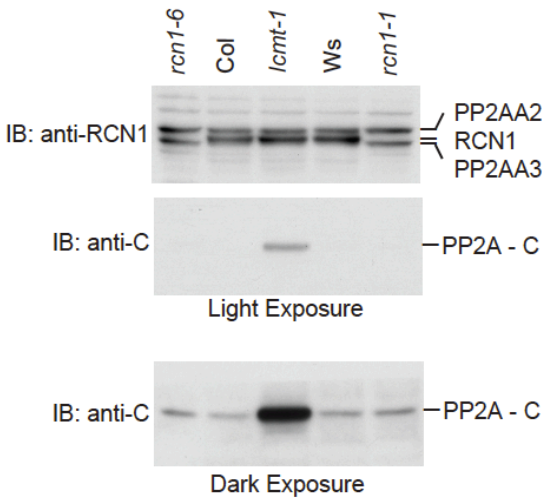
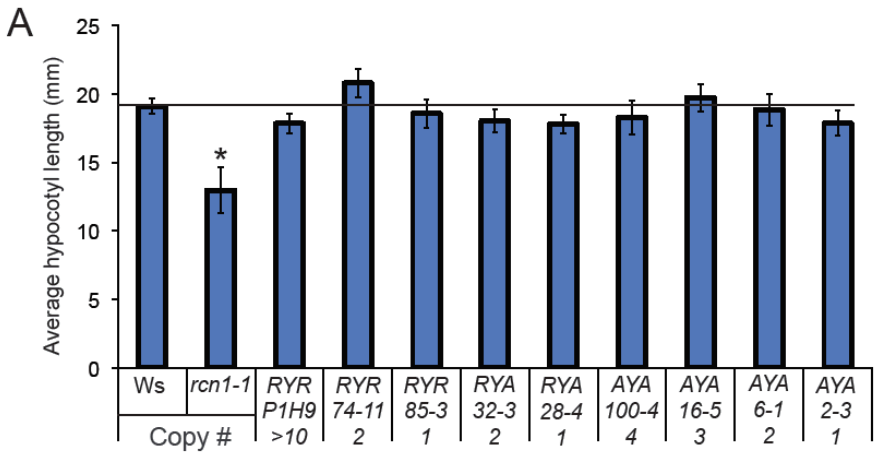
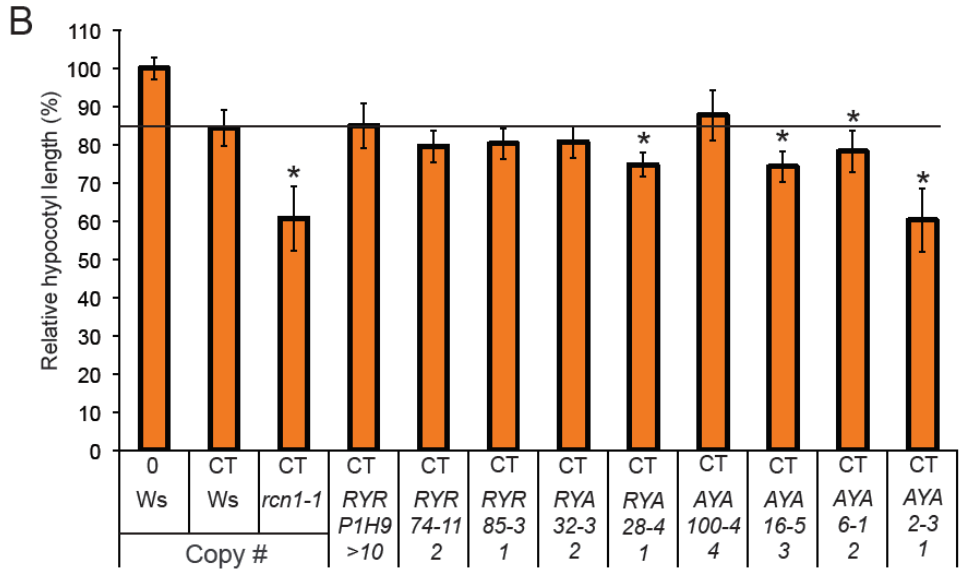



**Figure 4. Increased accumulation of PP2A-C but not PP2A-A subunits in *lcmt-1*.** Wild-type, *rcn1*, and *lcmt-1* seedlings were grown in the dark for 5 days and accumulation of PP2A-A and C subunits was analyzed by immunoblotting. Immunoblots were probed with anti-RCN1 antibodies to detect all three PP2A-A subunits and with anti-PP2A-C to detect total accumulation of PP2A-C. Light and dark exposures are of the same membrane probed with anti-PP2A-C antibodies (middle and bottom panels).


A
Average hypocotyl length (mm)
25
20
15
10
5
0
*
Ws
rcn1-1
RYR P1H9 >10
RYR 74-11 2
RYR 85-3 1
RYA 32-3 2
RYA 28-4 1
AYA 100-4 4
AYA 16-5 3
AYA 6-1 2
AYA 2-3 1
Copy #


B
Relative hypocotyl length (%)
110
100
90
80
70
60
50
40
30
20
10
0
*
0 Ws
CT Ws
CT rcn1-1
CT RYR P1H9 >10
CT RYR 74-11 2
CT RYR 85-3 1
CT RYA 32-3 2
CT RYA 28-4 1
CT AYA 100-4 4
CT AYA 16-5 3
CT AYA 6-1 2
CT AYA 2-3 1
Copy #

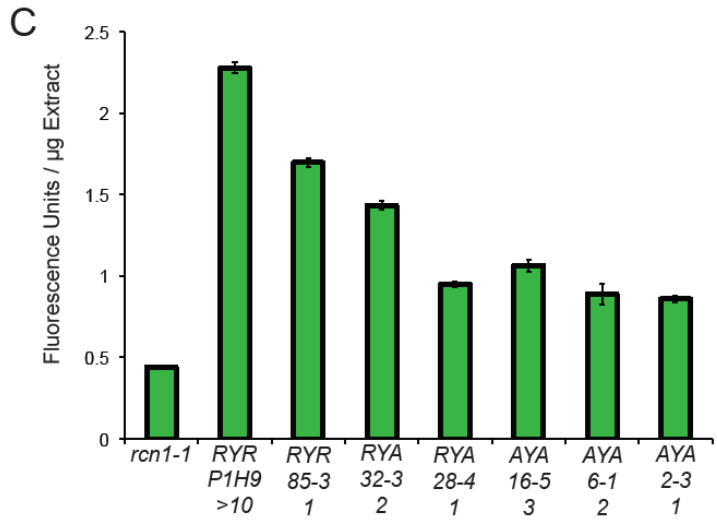

C
Fluorescence Units / µg Extract
2.5
2
1.5
1
0.5
0
rcn1-1
RYR P1H9 >10
RYR 85-3 1
RYA 32-3 2
RYA 28-4 1
AYA 16-5 3
AYA 6-1 2
AYA 2-3 1

**Figure 5. Hypocotyl elongation and resistance to inhibitor challenge are correlated with PP2A-A subunit expression in *rcn1-1* complementation assays.** (A) After 5 days of growth in the dark, the average hypocotyl length of wild-type (Ws), *rcn1-1,* and *rcn1-1* seedlings complemented with either YFP-RCN1 or YFP-PP2AA3 was analyzed. Insert copy numbers as determined by Southern analysis is shown below each name. Values shown represent average lengths; error bars show standard deviation ($n > 30$) (B) The hypocotyl elongation of same transgenic series from (A) was analyzed after growth in the presence of 3μM cantharidin (CT). Bars represent the relative hypocotyl elongation when grown on CT compared to growth in the absence of CT for each genotype; error bars show standard deviation; * indicates a significant difference from wild-type $p < 0.01$ ($n > 20$). (C) Extracts from transgenic plants were analyzed for YFP fluorescence to quantitate the accumulation of YFP-A subunit fusion proteins (see Materials and Methods).

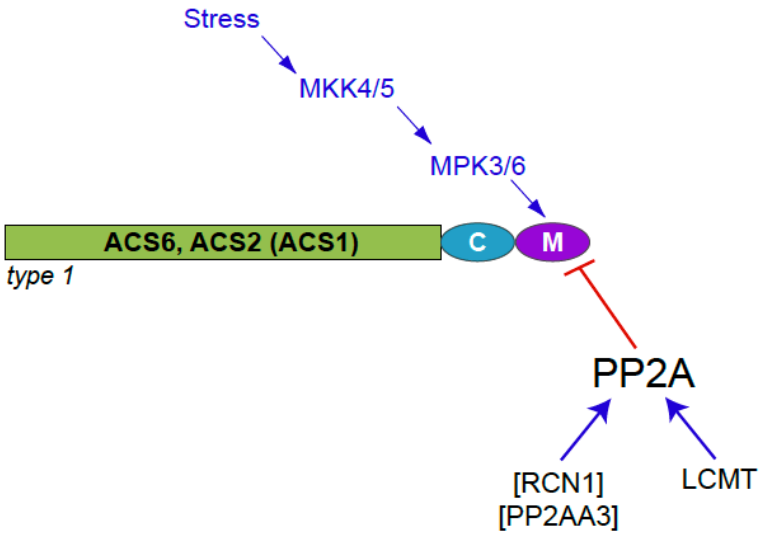



**Figure 6. PP2A regulation of type 1 ACS isozymes is modulated by LCMT and PP2A-A subunit levels.** Based on the analysis of *lcmt-1* mutant seedlings presented here, LCMT function appears necessary for generation ethylene regulating PP2A holoenzymes (see Figure 3). Additionally, the dosage of PP2A-A subunit appears to modulate PP2A activity to some degree. Increased expression of PP2A-A may increase PP2A activity slightly (see P1H9 in Figure 5B), while less PP2A-A accumulation appears detrimental to PP2A activity (see AYA series in Figure 5B).

# APPENDIX 2: THE QUEST FOR ADDITIONAL PP2A SUBSTRATES

Unpublished data

I performed all of the experiments presented in this appendix.

## INTRODUCTION

Previous observations of dark-grown *rcn1* seedlings uncovered a short-hypocotyl phenotype, which is due to ethylene overproduction (Garbers et al., 1996; Larsen and Cancel, 2003; Muday et al., 2006). The rate-limiting step in the ethylene biosynthetic pathway is conversion of S-adenosyl-L-methionine (SAM) to 1-aminocyclopropane-1-carboxylate (ACC), which is catalyzed by the ACC synthase (ACS) family of enzymes (Abeles, 1992). The N-terminal catalytic domains of the 9 Arabidopsis ACS isozymes are highly conserved. However, ACS enzymes are divided into three classes based on the types regulatory motifs present in their C-termini (reviewed in Argueso et al., 2007). Type 1 and 2 ACS isozymes are short-lived proteins and the overall rates of ethylene biosynthesis are regulated largely through modulating enzyme stability (Chae et al., 2003; Liu and Zhang, 2004; Figure 1). Little is known about the protein stability or post-translational regulation of type 3 enzymes.

There is a well-established link between ACS protein phosphorylation, enzyme stability, and ethylene production (Spanu et al., 1994; Liu and Zhang, 2004; Joo et al., 2008; Han et al., 2010; Kamiyoshihara et al., 2010). For these reasons, it was logical to pursue ACS isozymes as putative PP2A targets. We recently reported that the *rcn1* mutation results in hyperphosphorylation and increased enzyme stability for ACS6, a representative type 1 ACS isozyme (Skottke et al., 2011). Through biochemical and genetic analysis, we were able to demonstrate

that ACS6 (and presumably other type 1 ACS isozymes) are direct substrates of PP2A. However, two unexpected results from this work suggest that PP2A may exert additional regulatory control on ethylene biosynthesis through at least two distinct mechanisms.

The first unanticipated finding to come out of our analysis of PP2A regulation of ethylene biosynthesis was that PP2A regulates ACS isozyme stability differentially. Although PP2A normally functions to destabilize type 1 ACS isozymes through dephosphorylation of the C-terminal MAPK phosphorylation target sites, PP2A promotes the stability of type 2 ACS isozymes. This is demonstrated by the fact that ACS5, a representative type 2 ACS isozyme, is further destabilized in *rcn1* seedlings (Skottke et al., 2011; Figure 1). The mechanism through which PP2A regulates type 2 ACS isozymes in unknown.

The most parsimonious mechanistic hypothesis for how PP2A activity positively regulates the stability of type 2 ACS isozymes is through direct interaction. This type of regulation might involve dephosphorylation of the C-terminal putative CDPK target site. However, there is a precedent for PP2A-regulation mediated by binding of the target protein rather than catalytic function (Takemoto et al., 2009). PP2A could bind and non-catalytically protect type 2 ACS isozymes from turnover in a similar manner.

Another possible mechanism through which PP2A could positively regulate type 2 ACS isozyme stability involves ETO1 and two closely related homologs, EOL1 and EOL2. These proteins are bric-a-brac, tramtrack, broad complex (BTB) proteins that function as CULLIN3 based E3 ligase substrate adaptors to specifically target type 2 ACS isozymes for ubiquitination and degradation (Wang et al., 2004; Yoshida et al., 2005; Figure 1). Importantly, ETO1 and EOLs do not recognize either type 1 or type 3 ACS isozymes (Yoshida et al., 2006; Christians et al., 2009). In the ETO1-dependent model, PP2A activity would (either directly or indirectly) negatively regulate ETO1, thereby relieving type 2 ACS isozymes from proteasome-dependent turnover. EOL1 and EOL2 were recently identified as clients of the Arabidopsis 14-3-3-omega protein (Chang et al., 2009). Since 14-3-3 proteins bind phosphoproteins, this suggests that ETO1, EOL1, and EOL2 may be phosphorylated *in planta,* making them attractive candidates as phosphatase targets.

Our second unexpected finding involves a pathogen-sensing MAPK signaling cascade. Arabidopsis perceives bacterial pathogens through the *FLAGELLIN SENSING 2 (FLS2)* leucine-rich receptor-like kinase. FLS2 is involved in plant innate immunity by sensing flagellin, a component of eubacterial flagella, and activating a MAPK cascade composed of MEKK1, MKK4/5, and MPK3/6 to activate defense responses, including increased ethylene biosynthesis and the expression of defense-related genes (Gomez-Gomez and Boller, 2000; Asai et al., 2002; Gomez-Gomez and Boller, 2002). FLS2-mediated activation of

MPK3/6 results in phosphorylation of type 1 ACS isozymes, which increases ACS protein stability and ethylene biosynthesis (Liu and Zhang, 2004).

As part of our characterization of PP2A-mediated regulation of type 1 ACS isozymes, we assayed ethylene production in wild-type and *rcn1* seedlings in response to treatment with a flagellin peptide (flg22). We hypothesized that FLS2 stimulation would yield a smaller increase in ethylene biosynthesis in *rcn1* seedlings relative to wild-type because type 1 ACS isozymes are hyperphosphorylated in *rcn1* prior to flg22 treatment. Indeed, basal ethylene production was elevated in *rcn1* seedlings and treatment with flg22 resulted in a smaller increase in ethylene production compared to wild-type seedlings (Skottke et al., 2011). Surprisingly, *rcn1* seedlings failed to reach wild-type levels of ethylene production upon flg22 stimulation. This indicates there may be FLS2-MAPK signal attenuation in *rcn1* seedlings. Reduced PP2A activity could lead to FLS2-stimulated signal attenuation through aberrant MAPK activation and desensitization of the signaling cascade through a homeostatic mechanism. PP2A regulates MAPK signaling in mammals, lending credence to the notion that PP2A could alter FLS2-mediated signaling in Arabidopsis (Raabe and Rapp, 2003; Ramos, 2008).

In animals PP2A regulation of MAPK signaling occurs at many levels and is also bidirectional, inhibitory for certain components and activating for others. The mammalian Ras signaling cascade has been studied extensively. In this

cascade, the MEKK Raf, is localized to the membrane and maintained in an inactive state by phosphorylation of Ser259, which promotes the binding of an inhibitory 14-3-3 protein. An AC core dimer binds to Raf in the inactive state, and upon Ras activation, a PP2A-B subunit is incorporated and PP2A dephosphorylates Ser259, releasing the 14-3-3 protein and activating Raf (reviewed in Raabe and Rapp, 2003). In addition to positively regulating Raf by dephosphorylation, PP2A also activates the scaffold protein Ksr by dephosphorylating Ser297 and Ser392. Similar to the effect of Raf dephosphorylation, these Ksr dephosphorylation events also result in the release of 14-3-3 binding, which allows the Ksr scaffold containing MEK and ERK kinases to co-localize with activated Raf (Ory et al., 2003). PP2A also acts directly on MEK and ERK kinases, inactivating them through dephosphorylation (Junttila et al., 2008; Ramos, 2008). Therefore, predicting the outcome of PP2A-directed phosphoregulation of Arabidopsis MAPK cascade-mediated pathogen signaling is difficult.

If PP2A functions as an overall negative regulator of FLS2-MAPK signaling in Arabidopsis, it is possible that basal levels of MAPK signaling are increased in *rcn1* seedlings. If this were true, continued basal MAPK signaling could result in the dampening of MAPK-dependent signaling through homeostatic mechanisms. This may explain the attenuation of flg22-induced ethylene production in *rcn1* seedlings. We employed an in-gel kinase assay approach in order to query the basal activation status of MPK3 and MPK6 in wild-type and *rcn1* seedlings.

## RESULTS

### ACS5 interacts with PP2A *in vivo*

In order to test the hypothesis that PP2A interacts with type 2 ACS enzymes and regulates their protein stability directly, we employed a co-immunoprecipitation strategy. Using anti-RCN1 antibodies that recognize all three Arabidopsis PP2A-A subunits, PP2A complexes were immunoprecipitated from protein extracts isolated from wild-type plants expressing myc-ACS5 or myc-ACS5$^{eto2}$ as well as *rcn1-6* plants expressing myc-ACS5 (Figure 2). The immunoprecipitates were immunoblotted for ACS5 using anti-myc antibodies.

There is a strong enrichment of myc-ACS5 immunoprecipitated using anti-RCN1 antibodies compared to a no-antibody control (Figure 2A). This suggests that PP2A complexes interact with wild-type ACS5. The interaction between ACS5 and PP2A suggests that PP2A may regulate the stability of type 2 ACS isozymes directly. Surprisingly, myc-ACS5$^{eto2}$ was also co-immunoprecipitated with PP2A complexes (Figure 2B). The PP2A:myc-ACS5$^{eto2}$ interaction appears as robust as the PP2A:myc-ACS5 interaction (compare Figure 2A light with Figure 2B dark). The difference in basal accumulation of myc-ACS5 and myc-ACS5$^{eto2}$ protein is a result of differential induction between the two transgenic lines used and has been previously documented (Skottke et al., 2011). Interestingly, the interaction observed between PP2A and myc-ACS5 in wild-type plants was

completely abolished in the *rcn1* background, suggesting that myc-ACS5 does not stably interact with PP2AA2 or PP2AA3 in the absence of RCN1 (Figure 2C).

**MPK3 activity is increased in *rcn1* seedlings**

Given that PP2A is a well-established regulator of MAPK signaling in other organisms, we decided to compare the activation state of Arabidopsis MAPKs in wild-type and *rcn1* plants using an in-gel kinase assay (IGKA). In this procedure the model kinase substrate myelin basic protein (MBP) is embedded in an acrylamide gel, such that after separation of protein extracts by SDS-PAGE, local kinase activity can be assayed by the incorporation of radiolabeled phosphate on MBP. MPK3 and MPK6 migrate at approximately 42 and 44 kDa, respectively. Their identities are routinely confirmed by comparison to mpk3 or mpk6 mutant IGKA results, which lack either MPK3 or MPK6 activity, respectively. Kinase activities corresponding to MPK3 and MPK6 were detected and quantified using the MetaMorph software package (Figure 3). Interestingly, while the level of activated MPK6 was not altered in *rcn1* seedlings, there was a slight but reproducible increase in MPK3 activity in three independent experiments. The degree of crosstalk between MAPK signaling cascades in Arabidopsis is still poorly defined and our understanding of factors capable of modulating specific MAPK activity (i.e. MPK3 vs. MPK6) is still limited. Therefore it is difficult to predict if the increase in MPK3 activity in *rcn1* seedlings represents the loss of direct negative regulation by PP2A or misregulation of an upstream (indirect) event.

## DISCUSSION

### PP2A-dependent regulation of type 2 ACS isozymes

PP2A positively regulates the accumulation and stability of ACS5 and genetic data support a similar effect on ACS9 (Skottke et al., 2011). In wild-type plants, PP2A interacts with myc-ACS5 and myc-ACS5$^{eto2}$ in co-immunoprecipitation experiments (Figure 2). This suggests that PP2A may exert direct positive regulation on type 2 ACS isozymes. However, it is odd that although the wild-type ACS5 C-terminus is required for PP2A-dependent stabilization, PP2A interacts with both myc-ACS5 and myc-ACS5$^{eto2}$. This may indicate that while PP2A binds to both wild-type and *eto2* forms of ACS5, additional regulatory factors (e.g. ETO1) are required for modulating ACS protein stability.

PP2A appears to interact equally well with myc-ACS5 and myc-ACS5$^{eto2}$ (compare Figure 2A and Figure 2B). However, in this experiment, the higher basal accumulation of myc-ACS5 compared to myc-ACS5$^{eto2}$ makes a direct comparison of interaction strength with PP2A challenging (see Figure 2A, B total lanes). The transgenic myc-tagged ACS plant lines used in this experiment are Dexamethasone (Dex) inducible (Chae et al., 2003). Altering the concentration of Dex used for induction modulates myc-ACS protein accumulation (Skottke et al., 2011). In the co-immunoprecipitation experiment presented here, all wild-type transgenic plants were grown on media containing 25 nM Dex. Therefore the differences in accumulation of myc-ACS5 and myc-ACS5$^{eto2}$ reflect variation

between transgenic lines. In future experiments, the levels of Dex induction can be altered to normalize the starting accumulation of myc-ACS5 and myc-ACS5$^{eto2}$ protein. This will allow us to more accurately compare the relative strength of the interaction each protein has with PP2A. Reciprocal co-immunoprecipitations using anti-myc antibodies followed by immunoblotting with anti-RCN1 antibodies should also show whether PP2AA2 and PP2AA3 interact with type 2 ACS isozymes.

Interestingly, the interaction observed between PP2A and myc-ACS5 in wild-type plants was completely abolished in the *rcn1* background (Figure 2C). The PP2A-myc-ACS5 interaction could be lost in *rcn1* plants for several reasons. One possible explanation is that PP2AA2 and PP2AA3 are poor substitutes for RCN1 in recruiting the regulatory PP2A-B subunit(s) that mediate interaction with ACS5. If this were the case, then only PP2A complexes built around the RCN1 scaffold would be competent to incorporate the type 2 ACS isozyme-targeting PP2A-B subunit(s). In this scenario, the loss of RCN1 protein would directly impact the formation of the PP2A holoenzymes responsible for targeting type 2 ACS isozymes.

Alternatively, if all three Arabidopsis PP2A-A subunits are competent to form ethylene-regulating PP2A holoenzymes, then failure to form the specific holoenzyme that interacts with ACS5 in *rcn1* seedlings may be the result of PP2A defects upstream of PP2A-B subunit recruitment. If the decreased PP2A

activity in *rcn1* seedlings is the result of a defect in active PP2A-C subunit biogenesis / holoenzyme assembly, then the pool of active AC core dimers (PP2AA2:C and PP2AA3:C) may be limiting in *rcn1* plants. If the PP2A-B subunit(s) responsible for targeting ACS5 (or type 1 ACS isozymes in the case of ethylene overproduction) is low abundance or binds to AC core dimers poorly, then other PP2A-B subunits could preferentially incorporate in holoenzymes and outcompete the formation of ACS5-regulating holoenzymes. Analysis of PP2A-A:myc-ACS5 interaction in YFP-RCN1 and YFP-PP2AA3 transgenic lines will provide a direct test of the RCN1-requirement for type 2 ACS isozyme targeting. If myc-ACS5 interacts with YFP-PP2AA3 (in the absence of RCN1), then RCN1 is not specifically required for proper PP2A targeting of type 2 ACS isozymes, which would support the threshold model.

**PP2A-dependent regulation of MAPK signaling**

Our previous analysis of flg22-induced ethylene production indicated that FLS2-mediated pathogen signaling may be attenuated in *rcn1* seedlings (Skottke et al., 2011). After treatment with flg22, wild-type seedlings exhibit a drastic increase in ethylene production. In contrast, *rcn1* seedlings produce a higher basal level of ethylene, but maximal ethylene production in response to flg22 stimulation does not match that observed in wild-type plants. This indicates that a component of the FLS2-MAPK signaling cascade may be a target of PP2A regulation. The MAPK cascade downstream of FLS2 is a likely candidate for PP2A-mediated regulation since PP2A has been shown to regulate MAPK signaling in animals.

Our analysis of MPK3 and MPK6 activation in wild-type and *rcn1* seedlings indicates that basal MPK3 activity may be higher in *rcn1.* It is possible that increased basal MPK3 activity in *rcn1* plants partially desensitizes MAPK signaling, which may explain the reduced flg22 responsiveness in *rcn1* seedlings.

In addition to the well-characterized and understood *rcn1* ethylene overproduction phenotype, dark-grown *rcn1* seedlings also display increased responsiveness to ethylene (Larsen and Chang, 2001). It has been reported that a MPK3/6 containing MAPK signaling cascade functions downstream of, and inhibited by CTR1 (Ouaked et al., 2003; Yoo et al., 2008), although this is controversial (Ecker, 2004; Liu and Zhang, 2004; Xu et al., 2008; Bethke et al., 2009). Therefore, the role of MAPK signaling plays in ethylene signaling is still too ambiguous to know if increased MPK3 activity could account for any enhanced ethylene sensitivity in *rcn1* seedlings.

## MATERIALS AND METHODS

### Plant material

Transgenic plant lines harboring Dex-inducible myc-ACS5 and myc-ACS5eto2 constructs and the growth conditions used to induce ACS5 protein accumulation have been previously described (Chae et al., 2003; Skottke et al., 2011). The *mpk3-1* allele has been previously described as a knockout (Wang et al., 2007).

### Co-immunoprecipitations

Co-immunoprecipitations were performed as described previously (Skottke et al., 2011). Seedlings were grown in the dark for 5 days on media containing 25nM Dex (wild-type) or 100nM Dex (*rcn1*). Seedlings were ground in liquid nitrogen, thawed in Co-IP buffer (500mM Tris, pH 7.5, 100mM NaCl, 0.3M sucrose, 0.2% triton X-100, and protease inhibitors), and centrifuged for 15 minutes at 16,000 X g at 4°C. The supernatant was transferred to a new tube and protein concentration was adjusted to 250ug/150ul. For each IP, 250ug of protein extract was added to a tube with 200ul protein A agarose (Invitrogen) and 50ul of 1.5:100 antibody dilution. Tubes were incubated for 1 hour at 4°C, then centrifuged at 1,000 X g for 3 minutes. The supernatant was reserved and the pellet was washed twice for 5 minutes in Co-IP buffer. After centrifugation, proteins were eluted from the protein A agarose by gently washing in 4X loading buffer followed by boiling and immunoblotting.

**In-Gel Kinase Assays**

In-gel kinase assays were performed as described previously (Kim et al., 2003; Liu and Zhang, 2004). Protein Extracts were prepared in lysis buffer (20mM HEPES, pH 7.6, 100mM KCl, 1.5mM EGTA, 1mM EDTA, 10% glycerol, 0.5% Triton X-100, 20mM β-glycerolphosphate, 50mM NaF, 10mM βME, 1mM PMSF, 1mM benzamidine, 1mM $Na_3VO_4$, A+L and protein concentrations were normalized using the Bradford assay (Bio-rad). 30ug of protein extract were run per lane on a 10% acrylamide gel containing 1% w/v myelin basic protein (MBP). After the separation by SDS-PAGE, SDS was immediately washed out with 3-40minute washes at room temperature in SDS removing buffer (50mM Tris-Cl, pH 8.0, 20% isopropanol, 5mM NaF, 0.1mM $Na_3VO_4$) The gel was then washed for 1 hour at room temperature in Gel Washing Buffer 1 (50mM Tris-Cl, pH 8.0, 0.1mM EDTA, 5mM BME, 5mM NaF, 0.1mM $Na_3VO_4$) Followed by another 2-1 hour washes at room temperature in Gel Washing Buffer 2 (50mM Tris-Cl, pH 8.0, 0.1mM EDTA, 5mM βME, + 6M guanidine HCl, 5mM NaF, 0.1mM $Na_3VO_4$) Kinases were renatured with a series of washes in renaturation buffer (50mM Tris-Cl, pH 8.0, 0.1mM EDTA, 0.04% Tween 40, 1mM βME, 5mM NaF, 0.1mM $Na_3VO_4$) at 4°C (overnight). Kinase assay was performed by Incubating gel at 30°C for 30min with 30ml of Kinase Buffer (40mM Tris-Cl, pH 7.5, 20mM $MgCl_2$, 0.1mM EGTA) followed by an incubation at 30°C for 1h with 30ml of Reaction Buffer (40mM Tris-Cl, pH 7.5, 20mM $MgCl_2$, 0.1mM EGTA, 20uM cold ATP, 50-60uCi $\gamma^{32}$P-ATP). The reaction was stopped by washing gel in 5% TCA, 1% NaPPi until background radioactivity was low. The gel was Coomassie stained

and dried, followed by audioradiography analysis using a Phosphor imager screen. The relative intensities of MPK3 and MPK6 activity were normalized for protein loading (Coomassie image) and quantified using the MetaMorph software package.

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**FIGURES**

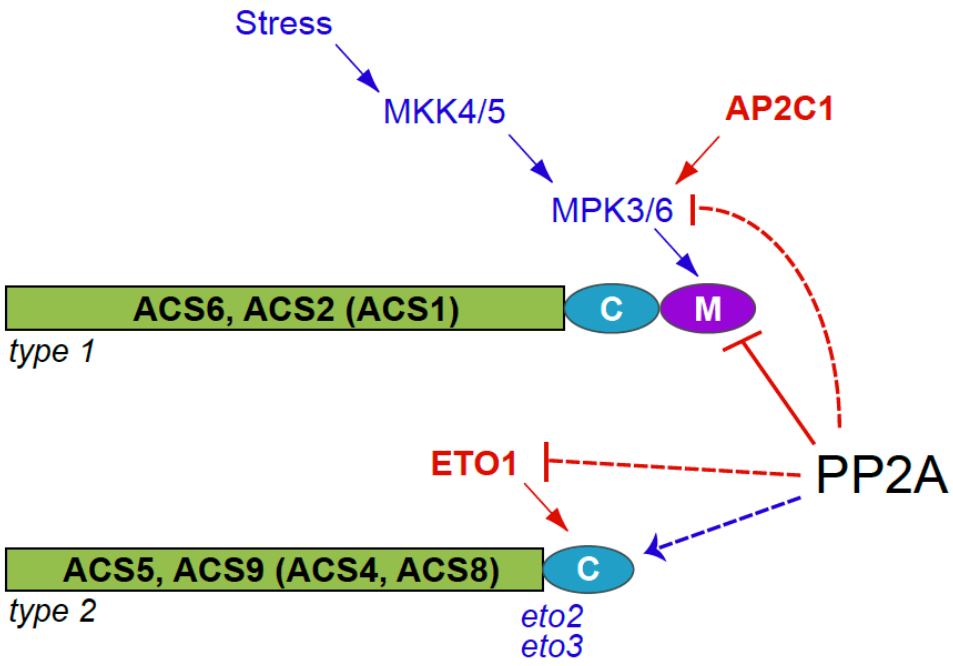


**Figure 1. Phospho-regulated stability of ACS isozymes.** Type 1 and 2 ACS isozymes are short-lived proteins whose stability is modulated through their C-terminal amino acid sequence motifs. Consensus sites for MAPK and CDPK phosphorylation are shown as purple and turquoise ovals, respectively. Several factors that affect the stability of type 1 and type 2 isozymes through action on C-terminal motifs are indicated, with positive regulators shown in blue and negative regulators shown in red. A stress-activated MAPK cascade containing MKK4/5 activates MPK3/6, which phosphorylate and stabilize type 1 ACS enzymes. PP2A counteracts this phosphostabilization by dephosphorylating the C-terminal MAPK phosphorylation sites in type 1 ACS isozymes. The ETO1 family of proteins specifically recognizes type 2 ACS isozymes and targets them for ubiquitination and subsequent degradation by the 26S proteasome. The *eto2* and *eto3* mutations are stabilizing alleles of ACS5 and ACS9, respectively. The *eto2* and *eto3* mutations alter the C-terminal ACS sequence that is required for recognition by ETO1. PP2A positively regulates type 2 ACS isozyme accumulation and stability through an unknown mechanism. Possible modes of PP2A-dependent type 2 ACS isozyme stabilization include (1) direct positive action on type 2 enzymes or (2) indirect action, possibly through negative regulation of ETO1.

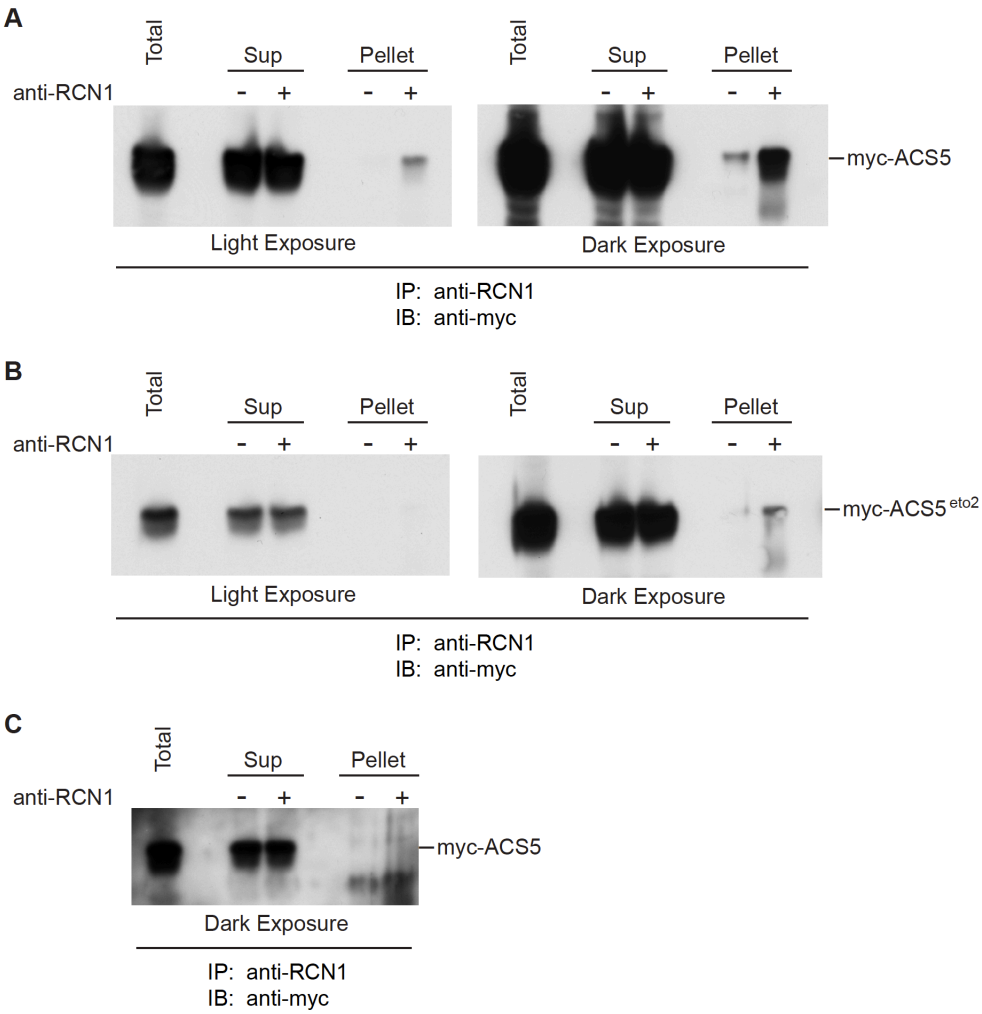


**Figure 2. PP2A interacts with ACS5 in wild-type but not *rcn1* plants.**
Protein extracts from wild-type plants expressing myc-ACS5 (A) or myc-ACS5$^{eto2}$ (B) or *rcn1* plants expressing myc-ACS5 (C) were subjected to immunoprecipitation (IP) with anti-RCN1 antibodies followed by immunoblotting (IB) with anti-myc antibodies to detect myc-ACS5 or myc-ACS5$^{eto2}$. Immunoprecipitates (Pellet fractions) were isolated and washed with buffer containing 0.1% Triton-X-100. Samples of the supernatant fractions (Sup) were also analyzed to test for depletion of the putative interactors. Control immunoprecipitates (lacking anti-RCN1 antibody) were also washed in buffer containing 0.1% Triton-X-100.

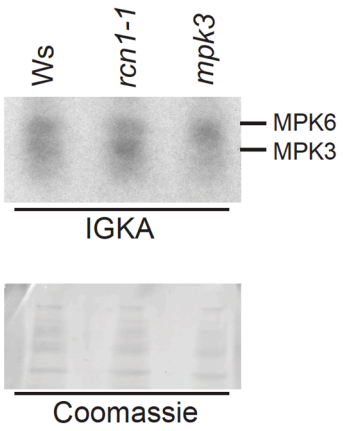


**Figure 3. MPK3 activity is increased in *rcn1* seedlings.** Proteins were extracted from 5-day-old dark-grown wild-type (Ws), *rcn1-1,* and *mpk3-1* seedlings directly in 4X SDS loading buffer. Proteins were separated by SDS-PAGE on a 10% acrylamide gel containing 1% w/v MPB substrate protein. IGKA was performed according to standard procedure as previously described (Kim et al., 2003; Liu and Zhang, 2004). After detection of kinase activity (upper panel) by autoradiography, the gel was Coomassie stained for a loading control. Relative intensity of MPK3 and MPK6 activation was compared to loading controls. There is no statistically significant difference between Ws and *rcn1-1* MPK6 activity, however, there is an 18% increase in MPK3 activity in *rcn1-1* seedlings. The *mpk3-1* extracts confirm the identity of the MPK3 activity band.