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Specificity of RCN1-mediated protein phosphatase 2A regulation in meristem organization and stress response in roots

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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
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). RCNI also functions as a transducer of ABA signals, acting upstream of ABA-induced increases in cytosolic Ca²⁺, 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 RCNI 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 RCNI, 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 stress response and meristem function.

RESULTS
Arabidopsis RCN1-YFP fusions retain biological activity
To facilitate our investigation of the biological specificity determinants for \textit{RCN1} function in plants, we constructed amino- and carboxy-terminal fusions of \textit{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, \textit{tpd3-1}. The \textit{tpd3-1} mutation confers temperature sensitivity and slow growth phenotypes that are complemented by the \textit{RCN1} cDNA (Garbers et al., 1996), as well as sensitivity to stress conditions such as nitrogen starvation and osmotic stress (Santhanam et al., 2004). \textit{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 \textit{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 \textit{RCN-YFP} and \textit{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 \textit{RCN1}-YFP fusion consistently exhibited a slightly slower SDS-PAGE migration when extracted from both yeast and plant cells (see below). The \textit{RCN1-YFP} fusion also complemented the \textit{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.

\textbf{YFP-RCN1 and YFP-PP2AA3 fusions rescue the \textit{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 \textit{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 \textit{rcn1-1} mutant plants. To test for biological function, we assayed for complementation of the \textit{rcn1} hypocotyl elongation defect (Figure 2B). In families segregating for an \textit{RCN\textsubscript{pro}:YFP-RCN1} (\textit{Ryr}) or
RCN<sup>pro:</sup>RCN1-YFP (RRY) fusion, YFP fluorescence segregated with rescued hypocotyl elongation, while hypocotyl lengths of YFP-negative siblings matched those of the <i>rcn1</i> 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 <i>PP2AA3</i> cDNA (Figure 2A). In one derivative the <i>YFP-PP2AA3</i> fusion remained under control of the <i>RCN1</i> promoter and 5' UTR (<i>RYA</i>), while in the second construct the <i>RCN1</i> upstream sequences were replaced with a 1.2 kbp fragment containing the <i>PP2AA3</i> leader (including a 312 bp intron), the intragenic region and the predicted 5' end of the next gene upstream (<i>AYA</i>). These constructs were designed to drive expression of the <i>YFP-PP2AA3</i> fusion in the <i>RCN1</i> and the <i>PP2AA3</i> domains, respectively. To avoid overexpression artifacts, we focused primarily on transformants carrying single copy T-DNAs in the <i>rcn1</i> background. We assayed the ability of these fusions to complement the hypocotyl elongation defect of <i>rcn1</i> seedlings (Figure 2C). Hypocotyl growth was fully restored in lines carrying the <i>YFP-PP2AA3</i> fusion under control of either the <i>RCN1</i> or <i>PP2AA3</i> promoter. Only a slight difference was observed between lines expressing <i>YFP-RCN</i> vs. <i>YFP-PP2AA3</i> fusions, and this difference was eliminated in lines carrying <i>YFP-PP2AA3</i> in two or more copies (Figure 2C, lines <i>rcn1 RYA-32</i>, <i>rcn1 AYA-6</i> and <i>rcn1 AYA-16</i>). 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 <i>rcn1</i> seedlings exhibit root tip disorganization phenotypes consistent with our hypothesis that increased basipetal auxin transport alters auxin distribution in <i>rcn1</i> 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, \textit{rcn1} seedlings showed disorganization indicating aberrant cell division patterns (Figure 3). Quiescent center (QC) and cortical/endodermal initial cells were difficult to distinguish in \textit{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 \textit{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 \textit{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 \textit{rcn1} allele in the Columbia genetic background showed similar defects in root tip organization.

We asked whether \textit{YFP-RCN} and \textit{YFP-PP2AA3} could rescue the abnormal morphology of \textit{rcn1} root tips (Figure 3). \textit{RRY} and \textit{RYS} 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 \textit{RYA} line (35\%; \(n = 23\)) and a representative \textit{AYA} line (26\%; \(n = 31\)). Similarly, a regular columella initial cell layer was present in all wild-type and \textit{RYS} root tips, with only 7\% exhibiting an abnormal columella initial cell number, while 53\% of \textit{rcn1-1} roots, 30\% of \textit{RYA} roots and 35\% of \textit{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 \textit{RCN1} function; \textit{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 \textit{PP2AA3} construct (\textit{PP2AA3\textsubscript{pro}, PP2AA3}). The native \textit{PP2AA3} construct also provided only weak complementation of \textit{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 \textit{rcn1 pp2aa2} and \textit{rcn1 pp2aa3} double mutants, but not in \textit{pp2aa2 pp2aa3} double mutant seedlings (Zhou et al., 2004). We asked whether root growth in the \textit{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 \textit{pp2aa2 pp2aa3} root tips, with clearly defined tiers and files of cells (see Supplemental Figure S2). In \textit{rcn1} root tips, columellar cell files and tiers often were irregular, indicating aberrant patterns of columella initial divisions. Overall columellar morphology in the \textit{pp2aa2 pp2aa3 rcn1/+} mutant was similar to that observed in the \textit{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 \textit{rcn1 pp2aa2} and \textit{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 \textit{pp2aa2 pp2aa3} background, the heterozygous RCN1 dose in \textit{pp2aa2 pp2aa3 rcn1/+} mutants supports more normal development than a homozygous PP2AA3 dose in the \textit{rcn1 pp2aa2} double mutant.

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

Abnormal embryogenesis in \textit{rcn1 pp2aa2} and \textit{rcn1 pp2aa3} plants (Zhou et al., 2004; Michniewicz et al., 2007) as well as enhancement of \textit{pin1} and \textit{pid} embryogenesis defects by \textit{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 \textit{rcn1} root tip defects. We and others have previously used
cantharidin to produce a phenocopy of rcnl 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 rcnl (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 rcnl-6, a T-DNA insertion allele (see Materials and Methods). RCN1 protein is undetectable in extracts of rcnl-6 seedlings, and the gross hypocotyl and root phenotypes of rcnl-6 seedlings are indistinguishable from those of the rcnl-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 rcnl-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 rcnl 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 rcnl 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 RCNI in specific cell types in seedlings. The requirement for RCNI 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 RCNI 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 rcnl 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 rcnl mutant is sufficient to promote normal hypocotyl elongation. Since increased ethylene synthesis in dark-grown rcnl 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 RCNI 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 Aα and Aβ 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 (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 \textit{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, \textit{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 \textit{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 \textit{PP2AC-2} function confers ABA hypersensitivity (Pernas et al., 2007), loss of \textit{RCN1} function results in reduced ABA sensitivity (Kwak et al., 2002). Paradoxically, both mutants show increased sensitivity to NaCl treatment. However, the \textit{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 \textit{rcn1}. These disparities indicate that the effect of \textit{rcn1} loss of function is unlikely to be mediated by a specific effect on regulation of PP2AC-2 activity.

The parallel stress sensitivity of \textit{rcn1} plants and \textit{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.
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 \textit{ADCI}_{pro}:\textit{RCN1}-YFP fusions for yeast were constructed using the triple-template PCR strategy (Tian et al., 2004), with the \textit{RCN1} cDNA (Garbers et al., 1996), the \textit{ADCI} 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 \textit{ADCI}_{pro}:\textit{RCN1}-YFP fusions were obtained and cloned into YEplac195 (Pitluk et al., 1995) using the Gateway and TOPO-XL systems (Invitrogen). \textit{SUP35:GFP} was the kind gift of T. Serio (Brown University). The genomic \textit{RCN1}-YFP and \textit{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 \textit{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 \textit{RCN1} transcript was amplified from Col-0 genomic DNA and substituted for the short promoter in the binary vector, yielding \textit{RCN}_{pro}:\textit{YFP-RCN} (\textit{RYR}). To generate the
RCN<sub>pro</sub>: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<sub>pro</sub>: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<sub>pro</sub>:YFP-PP2AA3. For PP2AA3<sub>pro</sub>: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<sup>-1</sup>) or FM4-64 (5µg ml<sup>-1</sup>) 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, ADHpro:RCN1, ADHpro:YFP-RCN1, ADHpro:RCN1-YFP and ADHpro:RCN1Y450H-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 OD600 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 -80°C. 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 µg/ml pepstatin, and 100 µg/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 (CEPDTRKTPDYFL) 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).

SUPPLEMENTAL MATERIAL

Supplemental Table 1  PCR primers
Supplemental Figure S1  Structural model of sequence differences between Arabidopsis A subunit isoforms
Supplemental Figure S2  RCN1 is haploinsufficient in the pp2aa2 pp2aa3 background
Supplemental Figure S3  Regulatory A subunit transgene products accumulate to native levels
Supplemental Figure S4  Accumulation and localization of YFP-PP2AA3 fusion proteins
Supplemental Figure S5  Expansion of cortical cells in salt-stressed rcn1 seedlings
Supplemental Figure S6  Normal stress sensitivity in pp2aa2 and pp2aa3 mutants
Supplemental Figure S7  Localization of a YFP-RCN1 fusion protein following NaCl treatment
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FIGURE LEGENDS

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 (Amerger, 1983) in tpd3-I 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 RCNP<sub>pro</sub>:YFP-PP2AA3 (RYA) construct, the RCN1 coding sequence was replaced with the PP2AA3 cDNA. The PP2AA3<sub>pro</sub>: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<sup>+</sup> or YFP<sup>−</sup>) 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 μm.

**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 μM 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 μM 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 μm (A-D, I - L) and 20 μm (E - H).
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 S5E - F). Scale bars, 25 µm (A – C, G – O) and 10 µm (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 1.** *RCN-YFP* fusions provide 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 *RCN1-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 constructs 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 amended with 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.
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