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*RCD1* and *SROI* Have Partially Redundant Developmental Roles

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The Paralogous Genes RADICAL-INDUCED CELL DEATH 1 and SIMILAR TO RCD ONE 1 Have Partially Redundant Functions During Arabidopsis thaliana Development

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ABSTRACT
RADICAL-INDUCED CELL DEATH1 (RCD1) and SIMILAR TO RCD ONE 1 (SRO1) are the only two proteins encoded in the Arabidopsis thaliana genome containing both a putative poly(ADP-ribose) polymerase (PARP) catalytic domain and a WWE protein-protein interaction domain, although similar proteins have been found in other eukaryotes. PARPs mediate attachment of ADP-ribose units from donor NAD\(^+\) molecules to target proteins and have been implicated in a number of processes including DNA repair, apoptosis, transcription, and chromatin remodelling. We have isolated mutants in both RCD1 and SRO1, rcd1-3 and sro1-1, respectively. rcd1-3 plants display phenotypic defects as reported for previously isolated alleles, most notably reduced stature. In addition, rcd1-3 mutants display a number of additional developmental defects in root architecture and maintenance of reproductive development. While single mutant sro1-1 plants are relatively normal, loss of a single dose of SRO1 in the rcd1-3 background increases the severity of several developmental defects, implying that these genes do share some functions. However, rcd1-3 and sro1-1 mutants behave differently in several developmental events and abiotic stress responses, suggesting that they also have distinct functions. Remarkably, rcd1-3; sro1-1 double mutants display severe defects in embryogenesis and post-embryonic development. This study shows that RCD1 and SRO1 are at least partially redundant and that they are essential genes for plant development.
INTRODUCTION

Poly(ADP-ribose) polymerases (PARPs) are a class of enzymes that post-translationally add negatively charged ADP-ribose (PAR) polymers synthesized from NAD⁺ to lysine residues on target proteins (Altmeyer et al., 2009). Depending on the specific PARP involved, one to hundreds of ADP-ribose units can be attached to the target (Kim et al., 2005). PARPs are found in all groups of eukaryotes and are characterized by the catalytic site, a β-alpha-loop-B-alpha NAD⁺ fold, also called the PARP signature (Ruf et al., 1996; Oliver et al., 2004). This family has been best characterized in humans, where there are eighteen family members with diverse functional domains outside of the PARP signature (Ame et al., 2004; Schreiber et al., 2006; Hassa and Hottiger, 2008). It is postulated that different PARPs participate in diverse events through these domains. However, it is unclear if all proteins with PARP signatures actually function as enzymes. For example, while tiPARP, PARP-9 and PARP-10 have replaced an important catalytic residue (Glu) with non-conserved residues (Aguiar et al., 2000; Ma et al., 2001; Yu et al., 2005), PARP-9 is enzymatically inactive (Aguiar et al., 2005) while ti-PARP is enzymatically active (Ma et al., 2001) and PARP-10 has transferase activity rather than polymerase activity, adding one ADP-ribose subunit to target proteins (Yu et al., 2005; Chou et al., 2006; Kleine et al., 2008).

PARPs have been implicated to be involved in DNA damage repair, cell death pathways, transcription and chromatin modification/remodelling (reviewed in (Kim et al., 2005; Schreiber et al., 2006; Hassa and Hottiger, 2008)). Human PARPs have been placed into five classes according to their functions: DNA-dependent PARPs, tankyrases, CCCH-type PARPs, macroPARPs, and a diverse orphan group with no known function(s) (Schreiber et al., 2006). The original PARPs identified, PARP-1 and PARP-2, are DNA-dependent PARPs that function in DNA-damage repair and are characterized by DNA binding, WGR, and PARP regulatory domains (PRD) N-terminal to their catalytic sites (Satoh and Lindahl, 1992; Trucco et al., 1998; Schreiber et al., 2002). Recent work has also implicated PARP-1 in other DNA associated functions such as transcriptional control, DNA methylation and modulation of promoter chromatin state by binding nucleosomes (Hassa et al., 2003; Carrillo et al., 2004; Fossati et al., 2006; Ju and Rosenfeld, 2006; Cohen-Armon et al., 2007; Ishiguro et al., 2007; Caiafa et al., 2008;
Choi et al., 2008; Guastafierro et al., 2008; Krishnakumar et al., 2008). The third member of the DNA-dependent PARPs, PARP-3, is similar to PARP-1 and PARP-2, except that it is missing a known DNA binding domain (Johansson, 1999). PARP-3 has been shown to associate with the centrosome, polycomb group proteins and DNA repair machinery (Augustin et al., 2003; Rouleau et al., 2007). A recent study in astrocytes suggests that all three DNA-dependent PARPs can act cooperatively during activation of this cell type (Phulwani and Kielian, 2008).

The other groups of PARPs have not been as extensively studied. The tankyrases are involved in telomere length control (Smith et al., 1998; Smith and de Lange, 2000; Cook et al., 2002) while CCCH-type PARPs, characterized by CCCH zinc fingers and a WWE domain in addition to the PARP catalytic domain, bind RNA, particularly viral RNA, through their zinc fingers and target the RNA for degradation (Gao et al., 2002; Guo et al., 2004; Guo et al., 2007; Kerns et al., 2008). The MacroPARPs contain macro domains (Aguiar et al., 2005), recently shown to bind ADP-ribose and PAR (Karras et al., 2005; Egloff et al., 2006); members of this family have been implicated in regulation of gene expression through interactions with transcriptional co-factors (Goenka and Boothby, 2006; Cho et al., 2009) and in protecting cells from DNA damage-induced apoptosis (Cho et al., 2009).

PARPs and the role of poly(ADP-ribosyl)ation has not been as well studied in plants as in animal systems. PARP inhibitor studies have demonstrated the involvement of PARPs in abiotic stress (Amor et al., 1998; De Block et al., 2005) and defence responses (Berglund et al., 1996; Adams-Phillips et al., 2008). A role for PARP activity in seeds to protect against genotoxic stress has also been inferred (Hunt and Gray, 2009).

Arabidopsis thaliana encodes nine putative PARP-encoding genes (Supplemental Fig. 1A). Orthologs of PARP-1 and PARP-2, the DNA-dependent PARPs, AtPARP1 (At4g02390) and AtPARP2 (At2g31320) respectively, have been identified (Lepiniec et al., 1995; Babiychuk et al., 1998). These genes have been implicated in DNA repair (Doucet-Chabeaud et al., 2001; De Block et al., 2005), and have been shown to be associated with chromosomes through their N-terminal zinc fingers (Babiychuk et al., 2001). AtPARP1 and AtPARP2 are up-regulated during Gemini virus infection, most likely in response to accumulation of nicked viral DNA forms (Ascencio-Ibanez et al.,
2008) and also accumulate under conditions of DNA stress (Culligan et al., 2006). The finding that two putative transcriptional co-activators can bind to the zinc fingers of AtPARP1 suggests that, like mammalian PARP-1, this protein may also be involved in regulation of gene expression in addition to its role in DNA repair (Storozhenko et al., 2001). Another gene, At5g22470, which is similar to the other two DNA dependent PARPs, is also found in Arabidopsis. No functional data on this gene has been published; however, it is highly expressed during seed development (Becerra et al., 2006). As expected, these three genes group together in a phylogenetic tree of flowering plant PARPs (Supplemental Fig. 1A). Down-regulation of AtPARP1 and AtPARP2 causes resistance to a number of abiotic stresses, including heat, cold, and drought (Amor et al., 1998; De Block et al., 2005; Vanderauwera et al., 2007), as does application of PARP inhibitors to plants (De Block et al., 2005). This effect may be mediated either directly through PARP targets or could be a consequence of altered NAD metabolism in the plants (Hashida et al., 2009).

No orthologs of the three other functional groups of PARPs known in humans have been identified. However, a group of four genes encoding relatively short proteins with the PARP signature but no other known functional domain(s) has been found (SRO2-SRO5; (Belles-Boix et al., 2000; Ahlfors et al., 2004)). This family consists of two gene pairs: SRO2/SRO3 and SRO4/SRO5 (Supplemental Fig. 1A). These pairs likely arose from the relatively recent genome duplication in Arabidopsis evolutionary history (Henry et al., 2006); the gene pairs are found in regions of synteny within the Arabidopsis genome (Plant Genome Duplication Database; http://chibba.agtec.uga.edu/duplication/index/home; (Tang et al., 2008)). These genes may be involved in stress signalling; SRO5 is necessary for response to both salt and oxidative stress (Borsani et al., 2005) and SRO2 is up regulated in chloroplastic ascorbic peroxidase mutants (Kangasjarvi et al., 2008).

Arabidopsis has two other genes, RADICAL-INDUCED CELL DEATH1 (RCD1) and SIMILAR TO RCD ONE 1 (SRO1), that encode putative PARPs with WWE domains N-terminal to the PARP signature (Belles-Boix et al., 2000; Ahlfors et al., 2004). This gene pair likely also arose from a genome duplication, as the two genes fall into two syntenic chromosomal regions (Supplemental Fig. 1A; Plant Genome Duplication
WWE domains are postulated to be protein-protein interaction domains and are found in proteins involved in the ubiquitin/proteosome pathway or in PARPs (Aravind, 2001), for example, in the animal CCCH-type PARPs (Katoh, 2003). Proteins with a similar domain structure to RCD1/SRO1 have been found in other plants including crop species such as rice (Supplemental Figure 1A; (Ahlfors et al., 2004)), suggesting that these proteins may play a conserved and vital role. In addition these proteins are similar to a PARP family member of unknown function from human, PARP11 (Supplemental Fig. 2; (Hakme et al., 2008)).

*RCD1* was originally identified as a stress response gene (Overmyer et al., 2000). It is involved in the response to several abiotic stresses, including ozone. All *rcd1* alleles confer dominant ozone hypersensitivity, although they seem to be recessive for other phenotypes (Ahlfors et al., 2004; Fujibe et al., 2004). *rcd1* mutants display accumulation of both ethylene and salicylic acid and altered expression of ethylene and abscisic acid responsive genes (Ahlfors et al., 2004). In addition, *rcd1* plants are hypersensitive to ozone (Overmyer et al., 2005) and conversely are resistant to UV-B (Fujibe et al., 2004). The phenotypic analysis suggests that *RCD1* may be a convergence point for several hormone-signalling pathways involved in the stress response and act both negatively and positively in stress responses (Ahlfors et al., 2004). *rcd1* mutants also display pleiotropic developmental defects including reduced stature, malformed leaves, and early flowering, consistent with the fact the gene is expressed in all tissues examined. These developmental defects do not seem to be associated with the defects in the hormonal signalling pathways examined to date (Ahlfors et al., 2004). Recently, phenotypes of *RCD1* overexpression transgenic lines have been reported. The overexpression caused dwarfing, normal response to an inducer of free radicals, methyl viologen, and hypersensitivity to ozone. Intriguingly, the *35S::RCD1* transgene was able to complement all tested *rcd1-2* phenotypes (Fujibe et al., 2006). A third *RCD1* allele, *rcd1-3*, has been identified (Katiyar-Agarwal et al., 2006), and displays similar phenotypes to *rcd1-1* and *rcd1-2*, although it has not been fully characterized. Taken together, the available data suggest that there is an optimal level of RCD1 activity/level for normal development and stress response.
The developmental phenotypes of \textit{rcd1} alleles have not been extensively analyzed and despite the significant sequence similarity, almost nothing is known about \textit{SRO1} function. This study presents data indicating that \textit{SRO1} possesses both unique and overlapping functions with \textit{RCD1} and that \textit{RCD1} and \textit{SRO1} function in previously unidentified developmental pathways, including embryogenesis.

\textbf{RESULTS}

\textbf{\textit{RCD1} and \textit{SRO1} are Paralogous Genes with Similar Expression Patterns}

\textit{RCD1} and \textit{SRO1} encode similar proteins that have 76\% similarity throughout their entire length (Fig. 1A; (Belles-Boix et al., 2000; Ahlfors et al., 2004)). Phylogenetic analysis indicates that these two genes are paralogs (Supplemental Fig. 1A), likely arising from a genome duplication (Plant Genome Duplication Database; http://chibba.agtec.uga.edu/duplication/index/home; (Tang et al., 2008)). This suggests that these two genes may be partially redundant. Functional redundancy would require that the two genes have similar expression patterns. \textit{RCD1} is expressed in all plant parts (Ahlfors et al., 2004). \textit{SRO1} is also expressed in all plant parts examined (Fig. 1B). \textit{SRO1} protein, like \textit{RCD1} (Fujibe et al., 2006), is localized to the plant nucleus (data not shown).

In order to compare the expression patterns of \textit{RCD1} and \textit{SRO1} during development in more detail, we utilized the AtGenExpress atlas (www.weigelworld.org/resources/microarray/AtGenExpress/; (Schmid et al., 2005)). This atlas compares the expression profiles of 22,746 probe sets on the Affymetrix ATH1 microarray from almost 80 diverse developmental samples. \textit{RCD1} and \textit{SRO1} were expressed in all samples; this was expected from our RT-PCR data and previous data (Fig. 1B; (Ahlfors et al., 2004)). For the most part, the pattern of transcript accumulation was similar between the two paralogous genes; however, \textit{SRO1} is consistently expressed at a lower level than \textit{RCD1} (Supplemental Fig. 3A). This similarity of expression pattern is reinforced by examining the correlation coefficients for expression of all gene expression vectors compared to that of \textit{SRO1} using Expression Angler on the Botany Array Resource (BAR) data set. (http://bar.utoronto.ca/ntools/cgi-bin/ntools_expression_angler.cgi; (Toufighi et al., 2005)). The data from BAR consist of
93 samples, with plant age, experiment type, tissue type, and treatment information appended. Using this tool, \textit{RCD1} is among the top three genes with the most similar expression pattern to that of \textit{SRO1} based on the Pearson Correlation Coefficient (r-value of at least 0.92; Supplemental Fig. 3C). Another of the top genes (\textit{At1g61420}) has been implicated in Arabidopsis innate immunity (Qutob et al., 2006), consistent with a function in stress response similar to \textit{RCD1} and \textit{SRO1}.

Since \textit{RCD1} has been implicated in abiotic stress response, \textit{RCD1} and \textit{SRO1} expression profiles in Arabidopsis samples challenged with abiotic stresses were compared, again using the AtGenExpress data (www.weigelworld.org/resources/microarray/AtGenExpress/; (Kilian et al., 2007)). As shown in Supplemental Fig. 3B, the two expression patterns are similar, with the level of \textit{SRO1} transcript lower than that of \textit{RCD1}. \textit{SRO1} expression level varies less in response to abiotic stress than \textit{RCD1}, but neither gene shows large changes in expression in response to any of the abiotic stresses tested by microarray. However, \textit{RCD1} transcript has been shown to accumulate under high light stress (Bechtold et al., 2008); \textit{SRO1} transcript has not been reported to do so. This suggests that transcription or transcript stability of \textit{RCD1} may be regulated during the response to specific abiotic stresses. The similarity in expression pattern between \textit{RCD1} and \textit{SRO1} is consistent with the hypothesis that these genes have at least partially redundant functions.

**Identification of Mutations in \textit{RCD1} and \textit{SRO1}**

We independently isolated a T-DNA insertion allele of \textit{RCD1}, \textit{rcd1-3}, which has previously been described (Katiyar-Agarwal et al., 2006). This allele can be complemented by a \textit{35S::RCD1} transgene (Fig. 5C), as previously reported for other \textit{rcd1} alleles (Ahlfors et al., 2004; Fujibe et al., 2006). We have identified a T-DNA insertion allele in \textit{SRO1}, \textit{sro1-1}. Both \textit{rcd1-3} and \textit{sro1-1} do not accumulate any detectable full-length transcript (Fig. 1C). However, \textit{sro1-1} appears to have partial transcripts arising both upstream and downstream of the T-DNA insertion site (Fig. 1D) while \textit{rcd1-3} also accumulates transcript 5’ to the insertion (data not shown), suggesting these alleles may be partial loss of function. However, an allele of \textit{RCD1}, \textit{rcd1-4}, an RNA null, is indistinguishable from \textit{rcd1-3} (J. Kangasjarvi, personal communication). Unlike \textit{rcd1-3}
mutants, sro1-1 mutants cannot be complemented by overexpressing SRO1 (data not shown). Complementation of sro1-1 mutants was achieved by transforming mutants with a 5kb genomic fragment including SRO1. This fragment was introduced into the rcd1-3; sro1-1 double mutant background, where the restoration of SRO1 function restores plants to an rcd1-3 mutant phenotype (Fig. 5F). Complementation was done in this background because sro1-1 has very mild phenotypes as a single mutant (see below).

**RCD1 and SRO1 Both Function in Abiotic Stress Response**

It has previously been reported that rcd1 is involved in response to a number of abiotic stresses (Overmyer et al., 2000; Ahlfors et al., 2004; Fujibe et al., 2004; Overmyer et al., 2005; Fujibe et al., 2006; Katiyar-Agarwal et al., 2006). The rcd1-3 allele behaves similarly to the previously described alleles by displaying increased resistance to chloroplastic ROS induced by paraquat (Table I). In contrast, rcd1-3 mutant plants are more sensitive to apoplastic ROS (H2O2; Table I). sro1-1 plants are also resistant to chloroplastic ROS (Table I), although to a lesser degree. Surprisingly, sro1-1 plants are resistant to apoplastic ROS (Table I), in contrast to the sensitivity of rcd1-3 plants. This suggests that these two genes are not always redundant in function, but may have independent functions under certain stress conditions. Similarly, rcd1-3 and sro1-1 display opposite salt stress phenotypes (Table II); rcd1 plants are salt sensitive while sro1 plants are resistant.

In contrast to the opposing roles in oxidative and salt stress, **RCD1 and SRO1** appear to act similarly in response to osmotic stress. rcd1-1 plants have been reported to display increased resistance to glucose (Ahlfors et al., 2004). In our hands, rcd1-3 plants display more resistance to both glucose and mannitol (Table III), suggesting this gene is involved in the response to osmotic stress, rather than being specifically involved in glucose signalling in the plant. Loss of SRO1 confers resistance to osmotic stress as does loss of RCD1 (Table III). The individual roles of RCD1 and SRO1 during stress response, therefore, appears to be complex.

**Analysis of Single Mutant Plants Indicates that RCD1 has a Larger Developmental Role Than SRO1**
The developmental defects of \textit{rcd1-3} and \textit{sro1-1} single mutants are of very different magnitudes. \textit{rcd1-3} plants display similar phenotypic defects to those reported for previously isolated alleles, \textit{rcd1-1} and \textit{rcd1-2} (Ahlfors et al., 2004; Fujibe et al., 2004; Fujibe et al., 2006) and \textit{rcd1-3} itself (Katiyar-Agarwal et al., 2006). These phenotypes include abnormally shaped leaves and mild early bolting (Fig. 2). Additional developmental defects were noted in \textit{rcd1-3} mutant plants, including small petals and abnormal hypocotyl elongation in the dark (data not shown) as well as changes in root architecture (Fig. 3). \textit{rcd1-3} mutants have shorter primary roots while lateral root number and length are increased. We observed the same trends in root growth in 6 day old, 16 day old and 21 day old seedlings (data not shown).

In contrast to \textit{rcd1} mutant plants, \textit{sro1-1} plants display only minor developmental defects, some of which are shared by \textit{rcd1} plants and some of which are not. Plant height, leaf shape and floral architecture are normal. Root architecture seems to be controlled by both \textit{RCD1} and \textit{SRO1}. In contrast to the \textit{rcd1-3} primary roots, those of \textit{sro1-1} are longer (Fig. 3); however, \textit{sro1-1} mutant seedlings have both an increased number and length of lateral roots, similar to \textit{rcd1-3}. Similarly to \textit{rcd1-3}, \textit{sro1-1} seedlings are wild type in appearance when germinated in the light but have a longer hypocotyl when germinated in the dark (data not shown). \textit{SRO1} does not always act in the same direction as \textit{RCD1}. For example, \textit{sro1-1} plants are mildly late flowering, although this is only significant in short days (Fig. 2), in contrast to \textit{rcd1-3}, which is early bolting. Taken together, our analysis of the single mutant phenotypes suggests that \textit{RCD1} has a larger developmental role than does \textit{SRO1} but that \textit{SRO1} is important for specific developmental events, such as root development.

\textbf{RCD1 Has a Role in Maintaining Reproductive Fate}

\textit{Arabidopsis thaliana} is a facultative long day plant. As such, it takes much longer to bolt and flower under short day conditions than under long day conditions. \textit{rcd1-3} plants bolt slightly early compared to wild type Columbia in both day length conditions as measured both by number of rosette leaves and days to bolting (Fig. 2). However, there is a defect in the transition to flowering after bolting that is particularly strong under non-inducing short day conditions. Upon bolting, wild type plants make 3-4 (long days)
or 5-6 (short day) cauline leaves before producing solitary flowers. In both day length conditions used in this study, rcd1-3 plants form aerial rosettes instead (Fig. 4A-E and data not shown), while wild type plants form none. Only one to two such rosettes are formed by rcd1-3 plants grown in long days before formation of cauline leaves with associated branches and then solitary flowers. In short days, up to 4-5 aerial rosettes and same number of aerial half-rosettes were formed before formation of cauline leaves. Only some rcd1-3 plants ever form flowers under short day conditions; however, these flowers never fully mature and do not open or form seed. In contrast, once sro1-1 flowers are formed in short days, they are normal and fertile (Fig. 4B).

The aerial rosettes formed on rcd1-3 plants are of two types: some completely encircle the circumference of the stem (Fig. 4C), suggesting they arise from the shoot apical meristem, and not from an axillary meristem, while others are formed in the axils of leaves, either cauline-like leaves (Fig. 4D) or in the axils of aerial rosette leaves (Fig. 4E). At a low frequency, both wild type and sro1-1 plants grown in short days have extra leaves forming in association with cauline leaves (Fig. 4F, G), however these structures never fully surround the stem and do not resemble aerial rosettes; they appear to arise from suppression of internode elongation.

The flowering repressor FLC is strongly expressed during vegetative development, but decreases upon reproductive induction and is not detected in inflorescences (Michaels and Amasino, 1999). Overexpression of FLC can cause formation of aerial rosettes (Wang et al., 2007). Consistent with the aerial rosettes formed by rcd1-3 plants in short days being caused by a reversion to vegetative growth, these structures express FLC at a high level compared to wild type cauline leaves associated with extra leaves (Fig. 4H). FLC expression is also misregulated in sro1-1 cauline leaves associated with extra leaves, but not to as high a level as in rcd1-3 (Fig. 4H). This may contribute to the mild late flowering of sro1-1 plants.

**RCD1 and SRO1 Control Plant Height and Vegetative Development**

rcd1-3 plants, like other characterized alleles, are significantly shorter than wild type; on the other hand, sro1-1 plants do not differ from wild type in height (Fig. 5B, Table IV). As single sro1-1 mutants have only mild developmental defects, it is possible
that the function of SRO1 is mostly complemented by the intact RCD1 locus but becomes necessary when RCD1 is absent or reduced. Therefore, reducing the dose of SRO1 may enhance the phenotype of rcdl. To test this, rcdl-3 was crossed to sro1-1 to construct rcdl-3; sro1-1 double mutants. In the F2 generation, plants with two novel phenotypes were observed. One of the novel phenotypes corresponded to the double mutant (see below) and one was determined to be rcdl-3/rcdl-3; sro1-1/+ by PCR genotyping. Specifically, the plants that were homozygous for rcdl and heterozygous for sro1 were significantly shorter than rcdl-3 single mutants (Fig. 5D; Table IV). Other developmental defects seen in rcdl-3 single mutants were also enhanced, including flower size, and lateral root number (data not shown). In contrast, rcdl-3/+; sro1-1/sro1-1 plants did not differ significantly from sro1-1 single mutants (data not shown).

The rcdl-3; sro1-1 double mutant plants were severely defective. On soil, only a very few double mutant plants were recovered even though they should occur at a frequency of one in sixteen. Even on growth media, the double mutant seed fail to germinate effectively, with only 39.5% (± 2.5) of mutant seeds germinating; this germination defect was not significantly improved by application of gibberellic acid (data not shown). The seedlings that form are small, mostly pale green, and malformed. In particular, the leaves are very small and sessile (Fig. 5A). Without supplementation of the growth media with sucrose, the seedlings will not progress beyond the cotyledon stage. Even with sugar supplementation, only a few seedlings will progress to adulthood and those plants have severe defects.

The adult double mutant plants are extremely dwarfed, typically only reaching approximately one to two inches in height, have a bushy growth habit, and very small malformed leaves (Fig. 5E; Table IV). In addition, they have small flowers and produce few seeds. To determine if the extreme dwarfness of rcdl-3; sro1-1 plants was caused by defects in cell size and/or cell number, inflorescence stems were examined by scanning electron microscopy (SEM). While the cells of rcdl-3 and wild type were not significantly different, the cells of the double mutant were smaller, with less elongation along the axis of the stem; cell size in the double mutant was also much more variable than that seen in the wild type (Fig. 6). When cell size was measured, rcdl-3; sro1-1 cells were only 50% as large as in wild type on average (10536 ± 4622 vs. 20148 ± 5887
pixels). *rcd1-3* cells are on average slightly larger than Columbia cells (22147 ± 5025). In addition to the cell elongation problem, the margins of the cells appear less smooth in *rcd1-3; sro1-1* than in wild type and *rcd1-3* (Fig. 6) and the *rcd1-3; sro1-1* stems contain fewer cells than wild type (data not shown).

Double mutant plants are abnormal from germination. Close examination of *rcd1-3; sro1-1* seedlings showed that the hypocotyls are much shortened or missing (Fig. 7A). In addition, little to no hypocotyl lengthening can be observed when the double mutant seedlings are exposed to exogenous gibberellin, suggesting these structures are missing in the mutants (Fig. 7B). The phenotype of the *rcd1-3; sro1-1* double mutant seedlings superficially resembles that of mutations in components of the brassinosteroid (BR) biosynthetic pathway, such as *det2* mutants (Noguchi et al., 1999); however, the double mutant seedlings do expand their leaves, in a manner similar to wild type, when exposed to exogenous BR (Fig. 7D). Consistent with *rcd1-3; sro1-1* leaves lacking petioles rather than having very short petioles, no visible lengthening of petioles could be seen in the *rcd1-3; sro1-1* seedlings exposed to BR unlike in wild type or the *rcd1-3* or *sro1-1* single mutant seedlings (Fig. 7D).

**RCD1 and SRO1 are Necessary for Embryo and Seed Development**

The presence of only a fraction of the expected double mutant plants among the F2 progeny of crosses between *rcd1-3* and *sro1-1* plants suggested that most *rcd1-3; sro1-1* mutants aborted prematurely. This proved to be the case. Although *rcd1-3; sro1-1* plants produce seeds, a large fraction of those seeds are not normal and none of the double mutant embryos or seedlings are normal. Seeds were classified into three classes: normal (Fig. 8A), Class I (misshapen; Fig. 8B) and Class II (misshapen and shrunken; Fig. 8C). More than 80% of seeds from a wild type plant are normally shaped. Similarly, *rcd1-3* and *sro1-1* single mutants produce at least 75% normal seeds (Table V). *sro1-1* plants produce slightly lower number of normal seeds than the wild type and *rcd1-3*. *rcd1-3; sro1-1* plants produce less than 10% normally shaped seeds. The seeds of the double mutants also look darker in color (Fig. 8B-C).

To determine the origin of the misshapen seeds produced by the double mutant, both ovule development and embryogenesis in the double mutant were examined. *rcd1-3;
sro1-1 ovules appear smaller than wild type and are not normally shaped (Fig. 9B). The misshapen integuments likely contribute most to the shape changes seen in Class I seeds. This conclusion is supported when seeds produced by rcdl-3; sro1-1 plants fertilized by wild type pollen are examined. Nearly all seed produced, although the embryos are heterozygous at both loci, are similar in appearance to Class I seeds (data not shown). However, the ovule defects alone do not explain the seed defects.

Embryo development in rcdl-3; sro1-1 mutants was examined next. Double mutant embryos from mature, but still green, seeds all have abnormal shapes and sizes, but there is considerable heterogeneity in the severity of the phenotype (Fig. 8E-F). Some embryos appear not to have progressed much past the globular stage. Most embryos developed further, but have abnormal shapes; the basal portions of the embryo (root and hypocotyl) are most affected. The root was often short and sometimes had no clearly differentiated root cap. The hypocotyls were most affected, being shortened or missing. This is consistent with the abnormal hypocotyls seen in germinated rcdl-3; sro1-1 seedlings (Fig. 7B). The shape and structure of the cotyledons was also not fully wild type. Overall the rcdl-3; sro1-1 embryos were smaller than wild type and did not bend as normal. In contrast the embryos of the rcdl-3 and sro1-1 single mutants are wild type in appearance (data not shown). Consistent with small embryos that do not fill the seed, developing seeds in young double mutant siliques appear to be relatively normal, although there is variation in size and shape (Fig. 8G), but by fruit maturity, the seeds have become very abnormal, most likely due to collapse of the seed around small embryos during desiccation (Fig. 8H).

To determine at what stage or stages developmental defects appear in rcdl-3; sro1-1, earlier embryonic stages were examined. Embryo development in the double mutant appears normal until the globular stage (Fig. 9D). From this stage on, variable defects are seen. By heart stage, embryos have become broader than wild type with misoriented cell divisions in the region of the developing hypocotyl (Fig. 9G, H). At maturity, the most normal rcdl-3; sro1-1 mutants have short hypocotyls and rounded cotyledons (Fig. 9J) and none of the mutant embryos fill the seed (Fig. 9J-N). In addition, there was often asymmetric growth of the cotyledons (Fig. 9L) and some embryos do not appear to have differentiated into recognizable structures (Fig. 9M).
Differences in Function Between \textit{RCD1} and \textit{SRO1} are Encoded in Both the Promoter and Coding Sequences of the Two Genes

As mentioned above, differences in the ability of the 35S promoter to complement mutations in \textit{RCD1} and \textit{SRO1} suggests that there are differences in transcription level and/or pattern, transcript stability, and/or protein stability between these two paralogs. However, expression analysis indicates that both \textit{RCD1} and \textit{SRO1} have broadly similar expression patterns (Supplemental Fig. 3) and, as demonstrated above, the two genes do share some functions. To access possible differences between \textit{RCD1} and \textit{SRO1}, we generated chimeric constructs between the genes and examined the ability of these constructs to complement both \textit{rcd1-3} and \textit{sro1-1} as well as examining the 35S constructs more closely (Fig. 10). 35S::\textit{SRO1} failed to complement \textit{rcd1-3}, similar to its inability to replace \textit{SRO1} function (data not shown). \textit{pRCD1::SRO1g} and \textit{pSRO1::RCD1} both complemented the \textit{rcd1-3} single mutant; however, the level of complementation by \textit{pRCD1::SRO1g} was more variable between independent transgenic lines, as visualized by plant height, with fewer lines conferring wild type height (Fig. 10B). Both of these chimeric constructs ameliorated the \textit{rcd1-3; sro1-1} phenotype, with \textit{pSRO1::RCD1} again having a higher activity as is the case in the \textit{rcd1-3} background (Fig. 10A). Although \textit{pSRO1::SRO1g} can fully complement \textit{sro1-1} (Fig. 5F), this construct cannot complement \textit{rcd1-3} either as a single mutant (data not shown) or in the double mutant background (Fig. 5F; Fig. 10B). Together these results reinforce the idea that, although \textit{RCD1} and \textit{SRO1} are paralogs, some differences in regulation or function have differentiated the two loci.

DISCUSSION

\textit{RCD1} and \textit{SRO1} Exhibit Partially Redundant But Not Identical Functions

In this study, we investigated the functions of \textit{RCD1} and its paralog \textit{SRO1} in \textit{Arabidopsis thaliana}. We discovered that these two genes play redundant roles during several aspects of development including embryogenesis, revealing a previously unknown role of \textit{RCD1} during embryonic development. \textit{SRO1} seems to have a more minor role compared to that of \textit{RCD1}; for example, the single \textit{sro1-1} mutation does not
affect plant height while *rcd1-3* does, but *sro1-1/+* can enhance the dwarf phenotype of *rcd1-3*. Thirdly, we found that *RCD1* is involved in stabilization of reproductive development.

*RCD1* and *SRO1* have very similar expression patterns; both genes are expressed in all plant organs at all times tested (Fig. 1B; Supplemental Fig. 3A) and show relatively little change in expression under various abiotic stresses (Supplemental Fig. 3B). Mutant analysis has demonstrated that both *RCD1* and *SRO1* are necessary for normal stress response. Since the transcripts of these genes don’t change drastically in response to such stresses, it is more likely that protein level, activity, and/or localization is likely to be altered. For example, it has been shown that RCD1 can traffic into the cytoplasm under salt stress (Katiyar-Agarwal et al., 2006).

The ability of various transgenes to complement the *rcd1-3* and *sro1-1* mutants suggest that *RCD1* and *SRO1* genes are not equivalent. Expression data that shows that the *RCD1* promoter drives a higher level of expression (Supplemental Fig. 3); this higher expression level presumably allows the *pRCD1::SRO1g* transgene to complement *rcd1-3* and *rcd1-3; sro1-1* plants while *pSRO1::SRO1g* can only complement *sro1-1*. However, the *SRO1* promoter can complement *rcd1-3* when driving *RCD1* but not *SRO1*. This suggests the coding regions of the two genes are not equivalent. This could be due to transcript instability conferred by the *SRO1* coding region, lower translation ability of the *SRO1* message, or differences in the stability or activity between RCD1 and SRO1 proteins. Similar variability in complementation ability of chimeric transgenes between *RCD1* and *SRO1* has been seen by others (J. Kangasjarvi, personal communication).

**Control of Reproductive Growth by *RCD1* and *SRO1***

Although *RCD1* and *SRO1* share many functions, the single mutants do not show identical phenotypes. In particular, the function of the two genes in control of the transition to reproductive growth in Arabidopsis is different. *sro1-1* plants flower slightly late compared to wild type plants under both long and short day conditions (Fig. 2); once the transition to reproductive growth has been made in these plants, it proceeds normally (Fig. 4). *rcd1-3* plants, on the other hand, bolt early under both day length conditions (Fig. 3). They do not retain reproductive identity correctly, however. Rather, *rcd1-3*
produce aerial rosettes, especially under short day conditions (Fig. 4). *rcd1-3; sro1-1* plants do not bolt under short day conditions, even after five months (data not shown), suggesting that *RCD1/SRO1* function is essential for the reproductive transition under non-inducing conditions. Further, this suggests that *SRO1* function in *rcd1-3* mutants allows bolting in short days.

The Arabidopsis ecotype Sy-0 has a distinct shoot morphology that includes formation of aerial rosettes (Poduska et al., 2003). *rcd1-3*’s short day phenotype is somewhat similar to the morphology of this accession. It has been determined that the *HUA2/ART1* allele present in the Sy-0 background causes enhanced expression of the floral repressor *FLC*, contributing to the formation of aerial rosettes. *35S::FLC* plants phenocopy the Sy-0 shoot morphology (Wang et al., 2007). The aerial rosettes of *rcd1-3* do express *FLC* at a higher level compared to wild type cauline leaves (Fig. 4H), suggesting that misexpression of this gene may contribute to formation of aerial rosettes seen in *rcd1-3* plants. However, Sy-0 and *35S::FLC* plants both produce many vegetative leaves before bolting and formation of aerial rosettes, in contrast to the mild reduction of rosette leaf number seen in *rcd1-3* plants (Fig. 3), suggesting there may be additional factors involved. The Sy-0 accession has an active *FRIGIDA (FRI)* allele, which Columbia does not (Poduska et al., 2003). Crossing the *rcd1-3* allele into a background with an active *FRI* allele may lead to late bolting as well as aerial rosette formation.

The genetically separable bolting versus flowering seen in *rcd1-3* mutants suggests that a two-phase transition during flowering: first an inflorescence-producing phase then a flower-producing phase. Loss of *RCD1* function would interfere with the transition from inflorescence to flowering. Hempel and Feldman provided evidence for a single-phase transition in Arabidopsis in which existing leaf primordia could be transformed to flowers upon floral induction (acropetal development) whereas paraclades were formed from later arising primordia (Hempel, 1994); however, later work using Columbia grown under short day conditions supported a two-step phase transition under non-inducing conditions (i.e. paraclades arise first and flowers second) and further suggested that flowering time mutations have different effects on phase transition during flowering under different regimes (Suh et al., 2003). Our data support such a two-phase transition and suggest that *RCD1* functions in the transition from inflorescence
production to flower production, perhaps through control of FLC expression. FLC expression is controlled on many levels, including epigenetic modification of chromatin (Dennis and Peacock, 2007), transcriptional activation (Kim et al., 2006) and mRNA processing (Xing et al., 2008; Liu et al., 2007). Poly(ADP-ribosyl)ation has been implicated in all three of these processes in other systems (Quenet et al., 2009; Hakme et al., 2008; Ji and Tulin, 2009), therefore, it is difficult to assign RCD1 a specific function in control FLC expression without further experimentation.

RCD1 and SRO1 Are Essential for Normal Embryogenesis

RCD1 mutants were originally identified on the basis of their response to the abiotic stress ozone (Ahlfors et al., 2004). While rcd1 mutants have a number of developmental defects, most noticeably in stature, there was no lethality associated with loss of RCD1 function. Similarly, we have shown that sro1-1 mutants have only subtle developmental defects. However, rcd1-3; sro1-1 plants do not all proceed successfully through embryogenesis, demonstrating that these genes are essential for embryogenesis and act redundantly during this developmental stage.

The defects seen in rcd1-3; sro1-1 mutant embryos are completely expressive, as all have some abnormality, but are heterogeneous in severity, ranging from an apparent arrest at the globular stage to formation of mature embryos that can germinate and survive (Fig. 8, 9), although the plants formed are abnormal. This heterogeneity in phenotype could be due to the nature of the sro1-1 allele itself. This allele is not an RNA null mutation (Fig. 1D), and there likely remains some level of gene function present. The amount of function present may vary in individual embryos, leading to defects at various steps in embryogenesis. If a null allele in the SRO1 locus is identified, it may have a more severe phenotype, both as a single mutation and in combination with rcd1-3. It is not surprising that both members of a pair of paralogous genes need to be mutated before a function in embryogenesis is seen. At least 35 gene pairs that do not have clear embryonic lethality as single mutants do encode essential functions as indicated by lethality of double mutant combinations (http://www.seedgenes.org/7R_Double_Mutant_List.html; (Tzafrir et al., 2003)).
The heterogeneous nature of the embryonic defects in rcd1-3; sro1-1 plants makes determining the process or processes these two genes are involved with more difficult to determine. However, since embryogenesis proceeds normally through the globular stage (Fig. 9D), it is likely that delineation of the basic axes of the embryo is normal, as this is completed by the end of the globular stage (Jenik et al., 2007). In general, the basal region of the rcd1-3; sro1-1 embryos is more severely disrupted than the apical region, where the two cotyledons are usually relatively normal (Fig. 8, 9). The hypocotyls of the mutant embryos, in particular, are shortened, as are the roots to a lesser extent. Most of the embryos that have progressed past the globular stage do have both a root and shoot apical meristems, again suggesting that the apical-basal polarity of the embryo is intact. This suggests that the function(s) of RCD1/SRO1 may be most necessary for hypocotyl formation. This conclusion is further supported by the appearance of the rcd1-3; sro1-1 seedlings that do germinate. These seedlings have no hypocotyls or very shortened hypocotyls (Fig. 7). In general these defects suggest that there may be a deficit in auxin signalling or transport, as this plant hormone is essential for hypocotyl formation (Jenik and Barton, 2005). This hypothesis is currently being tested.

**Possible mechanisms of RCD1 and SRO1 action**

RCD1 has been found to bind a number of Arabidopsis transcription factors by yeast two-hybrid assay (Belles-Boix et al., 2000; Ahlfors et al., 2004). We propose that RCD1 and SRO1 act by binding specific transcription factors and associating with chromatin through them. RCD1 is normally localized in the nucleus (Fujibe et al., 2006), supporting such a role. Once there, they poly(ADP-ribosyl)ate target protein(s) to regulate transcription. The PARP catalytic domains of RCD1 and SRO1 retain several important conserved residues that are present in the human, murine, chicken, maize and Arabidopsis PARPs (Ruf et al., 1996; Ruf et al., 1998; Ruf et al., 1998; Ame et al., 2004; Kinoshita et al., 2004; Oliver et al., 2004; Bellocchi et al., 2005). Importantly, the residues necessary to form the donor site (G347, L348, S375, Y378) are present in the two proteins as is the residue, Y378, necessary for forming the acceptor site (Ruf et al., 1996; Oliver et al., 2004). The presence of these residues suggests that RCD1 and SRO1
function as PARPs, although this will need to be tested experimentally. Target proteins are likely to include RCD1 and SRO1 themselves, as most active PARPs automodify themselves (Mendoza-Alvarez and Alvarez-Gonzalez, 1993; Lindahl et al., 1995; Mendoza-Alvarez and Alvarez-Gonzalez, 1999). Substrates could also include the transcription factors RCD1 and SRO1 bind to, histones, transcriptional co-activators, and/or chromatin remodelling factors. Examples of such activity by PARPs in other systems is abundant in the literature (for example, (Goenka et al., 2007; Krishnakumar et al., 2008; Sala et al., 2008)). Given the pleiotropic nature of the \textit{rcd1-3; sro1-1} mutant phenotype, it is likely that the RCD1 and SRO1 proteins have multiple targets that vary both temporally and spatially.

The transcription factors that bind to RCD1 in yeast two-hybrid assays have demonstrated roles in stress response. For example, RCD1 can bind to STO, a transcription factor involved in salt stress response (Belles-Boix et al., 2000). This correlates well with the salt sensitivity of \textit{rcd1} mutants (Table II; (Ahlfors et al., 2004; Katiyar-Agarwal et al., 2006)). However, it was also found that RCD1 interacts with the plasma membrane Na\(^+\)/H\(^+\) antiporter SOS1 under salt stress conditions (Katiyar-Agarwal et al., 2006); under this stress, some of the protein appears to accumulate in the cytoplasm where it interacts with the cytoplasmic tail of SOS1. This suggests that RCD1 may also function outside of the nucleus.

Surprisingly, the region of RCD1 shown to bind to the transcription factors in yeast is the very C-terminal region, consisting of the end of the PARP catalytic domain and sequences beyond that (Belles-Boix et al., 2000) and not the WWE domain, although this domain is known to mediate protein-protein interactions. This suggests that the WWE domain brings in other protein(s) into complex with RCD1/SRO1 and possible transcription factor binding partners. These other proteins might be the actual targets of ADP-ribosylation by RCD1/SRO1. While association of RCD1 with transcription factors that mediate stress response may explain the stress phenotypes seen in \textit{rcd1} mutants, to date none of the identified binding partners can explain the developmental defects, especially those of \textit{rcd1-3; sro1-1} embryos. This suggests that other binding partners need to be identified. Recent unpublished work has identified RCD1 binding to transcription factors implicated in development (J. Kangasjarvi, personal...
communication); of particular interest, RCD1 can bind to IAA11, an auxin-responsive transcription factor that is expressed in embryos (Genevestigator; (Zimmermann et al., 2005)). Given that the embryonic defects seen in rcd1-3; sro1-1 embryos suggest auxin defects, interaction with this transcription factor may help explain some of the developmental defects.

Consistent with a role of RCD1 in abiotic stress response, microarray expression analysis of the rcd1–1 mutant identified genes involved in such response as having changed levels of expression in the mutant (Ahlfors et al., 2004). Upregulated genes include At3g22370, which encodes Alternative Oxidase1a shown to be necessary for normal response to light and drought stress (Giraud et al., 2008) and COR47 encoding a dehydrin also implicated in drought stress (Mouillon et al., 2008). The genes downregulated in an rcdl background included three other genes encoding dehydrins, ATDI8 (Puhakainen et al., 2004), ERD10 (Kovacs et al., 2008), and RAB18 (Lang and Palva, 1992). All of the proteins encoded by these genes are involved in the response to problems in either redox balance in the cell and/or problems in protein folding under stress conditions. Changes of these transcripts can help explain changes in abiotic stress response seen in the rcd1-3 mutants, but not necessarily the developmental defects. It is also difficult to determine if changes in expression of stress response genes is a direct effect of the loss of RCD1 function, or a consequence of altered redox metabolism in mutant plants, since loss of PARP function can lead to accumulation of NAD and oxidative stress (Hashida et al., 2009; Formentini et al., 2009). Therefore, more work will need to be done before the mechanism of action of RCD1 and SRO1 can be determined.

Conclusions

The putative PARPs RCD1 and SRO1 represent an important class of regulatory molecules with roles in embryogenesis, vegetative and reproductive development and abiotic stress responses in Arabidopsis thaliana. Among the nine putative PARPs identified in the Arabidopsis genome, RCD1 and SRO1 are most similar to each other and we show that they exhibit both redundant and divergent functions during development and stress response. Our study provides important insights into the complexity in the relationship between two highly similar paralogous genes. In addition, orthologs of
RCD1 and SRO1 are present throughout the flowering plants and similar proteins are found all the way to humans. Therefore, it is likely that information generated on the molecular mechanism of action of these genes in Arabidopsis will be applicable to other systems as well.

MATERIALS AND METHODS

Phylogenetic Analysis

Sequences of the Arabidopsis thaliana PARP catalytic domain-containing proteins were retrieved from NCBI (http://www.ncbi.nim.nih.gov/Tools/) and the PARP catalytic regions identified using Pfam version 23.0 (http://pfam.sanger.ac.uk/; (Finn et al., 2008; Coggill et al., 2008)). Sequences from Angiosperm species were identified and retrieved using NCBI BLAST searches. The PARP catalytic regions within these proteins were identified using Pfam. These regions were isolated using Perl scripts from the “Wildcat Toolbox” (http://proteomics.arizona.edu/toolbox.html; (Haynes et al., 2006)). ClustalX (http://www.clustal.org; (Larkin et al., 2007)) was used to generate alignments of the PARP catalytic regions. Phylogenetic trees were generated using ClustalX and the Neighbour Joining Method excluding gaps. Bootstrap values are included in the tree (Supplemental Fig. 1A). The tree was plotted using NJplot software (http://pbil.univ-lyon1.fr/software/njplot.html; (Perriere and Gouy, 1996)).

Plant Growth Conditions, Mutant Identification and Genetic Crosses

The rcd1-3 allele has been previously described (Katiyar-Agarwal et al., 2006) and was obtained from the Arabidopsis Biological Resource Center (ABRC; http://www.arabidopsis.org/abrc/). The sro1-1 allele was also obtained from the ABRC and is a T-DNA insertion from the SALK collection (SALK_126383; http://signal.salk.edu/; (Alonso et al., 2003)). rcd1-3; sro1-1 double mutant lines were created by crossing the respective homozygous mutants. F1 plants were confirmed by PCR genotyping (see below) and allowed to self-pollinate. Double mutant lines were identified from the F2 population by segregation of a novel phenotype and confirmed by PCR genotyping. All genotypes were confirmed in the F3 generation.
Arabidopsis thaliana seeds were vernalized for 3 to 5 days and were grown on Fafard-2 Mix soil (55% peat, perlite and vermiculite) with subirrigation at 22°C with 50% relative humidity under long-day (16 h, 80 µmol m⁻² s⁻¹) irradiance in controlled growth chambers (Enconair Ecological Chambers Inc., Manitoba, Canada) or growth rooms under similar conditions. Short day conditions varied only in that the illumination was limited to 8 hours of approximately 45-µmol m⁻² s⁻¹. Starting two weeks after planting, flats were regularly watered with fertilizer water (Peters Professional 20-10-20 Peat-Lite special fertilizer, Scotts, Marysville, Ohio) with a final concentration of 180 ppm. Plants studied for height and other phenotypes were grown side by side under identical conditions.

Seeds used in germination and root growth assays were sterilized with 70% ethanol followed by 10% (v/v) hypochlorite (bleach) and placed on Murashige & Skoog (MS) media (RPI Corp.) agar plates with indicated amount of sucrose (see below), incubated in the dark for three days at 4°C, and then grown under long day conditions at 22°C in a CU-36L Plant Growth Chamber (Percival Scientific Inc., Perry, IA).

Genotyping and Cloning
For genotyping, genomic DNA was extracted from the seedlings or leaves by crushing plant material in liquid nitrogen and extracting with Urea Extraction Buffer (7.0M Urea, 0.31M NaCl, 0.05M Tris-Cl pH-8, 0.02M EDTA pH-8, 1% (w/v) Sarcosine) followed by phenol:chloroform:isoamyl alcohol (25:24:1 v/v/v) extraction. The genomic DNA was used as template in PCR reactions; the primers used to detect the presence of the T-DNA insertion in rcd1-3 were S383-LP and LBb1, and for the sro1-1 insertion, S432-RP and LBb1 (Supplemental Table I). The wild type locus was amplified with primers S383-LP and S383-RP and S432-LP and S432-RP, respectively. The sequence of primer LBb1 was obtained from the SALK website (http://signal.salk.edu/; Supplemental Table I). PCR was done using Biolase Red DNA Polymerase (Bioline) on a conventional PCR machine (Bio-Rad- icer Thermal Cycler).

Cloning and Arabidopsis Transformation
*RCD1* cDNA (Stock # U11347) was obtained from the ABRC. Amplification of the *RCD1* coding sequence was done using the cDNA as template and the primers RCD1-F and RCD1-R (Supplemental Table I). PCR was done using Platinum® *Pfx* DNA Polymerase (Invitrogen). The PCR product was introduced into the pENTR-D Gateway® entry vector (Invitrogen) to form pENTRD-RCD1 and sequenced at Plant-Microbe Genomics Facility, The Ohio State University, Columbus, Ohio. pENTRD-RCD1 was then recombined with pGWB2 (Gateway® destination vector, a gift from T. Nakagawa, Shimane University, Matsue 690, Japan) to form p35S::RCD1, which was then introduced into *Agrobacterium tumefaciens* strain GV3101 by electroporation. *rcd1-3* plants were transformed using the floral dip method (Clough and Bent, 1998). Transformants were selected on MS agar plates with hygromycin (30µg/ml). The height of three independent transgenic lines was compared to that of wild type and the untransformed *rcd1-3* mutant to determine if the transgene complemented the mutant (Table IV).

Complementation of *sro1-1* mutants was achieved by transforming *sro1-1* mutants with a 5kb genomic fragment that includes *SRO1*. This fragment was cloned by PCR using Columbia genomic DNA (prepared as above) as template and the primers SRO1-221-F and SRO1-221-R (Supplemental Table I). PCR was performed using *Pfu* DNA polymerase (Stratagene). The PCR product was then recombined with the Gateway® entry vector pDONR221 (Invitrogen) to form pDONR-gSRO1 and sequenced as above. pDONR-SRO1g was recombined with pGWB1 Gateway® destination vector and introduced into Agrobacterium as above to form pSRO1::SRO1g. Transformation and selection of transgenic plants was done as with *RCD1*. Complementation of *sro1-1* was achieved in the *rcd1-3* background.

Chimeric constructs of pRCD1::SRO1g and pSRO1::RCD1 were generated by using the MultiSite Gateway® Pro 2.0 Kit (Invitrogen). The promoters of *RCD1* (pRCD1) and *SRO1* (pSRO1) were amplified using *Pfu Ultra* DNA polymerase (Stratagene) and the primers pRCD1-F & pRCD1-R, and pSRO1-F & pSRO1-R, respectively (Supplemental Table I). The PCR products were then recombined with the pDONR P1-P5r Gateway® vector (Invitrogen) to form Entry clones pDONR-pRCD1 and pDONR-pSRO1, respectively. *RCD1* coding sequence (RCD1) and *SRO1* gene (SRO1g) were
amplified using Platinum® Pfx DNA Polymerase (Invitrogen) and the primers RCD1c-F & RCD1c-R, and SRO1g-F & SRO1g-R, respectively (Supplemental Table I). The PCR products were then recombined with the pDONR P5-P2 Gateway® vector (Invitrogen) to form Entry clones pDONR-RCD1 and pDONR-SRO1g. pDONR-pRCD1 with pDONR-SRO1g and pDONR-pSRO1 with pDONR-RCD1, were recombined separately with pGWB1 Gateway® destination vector to form pRCD1::SRO1g and pSRO1::RCD1. Agrobacterium and plant transformation was done as described above. The chimeric constructs were used to complement both rcd1-3 and rcd1-3; sro1-1.

RNA Isolation and RT-PCR
RNA isolation was done from various plant tissues as described in the text using Trizol according to manufacturer’s instructions (Invitrogen). cDNA was prepared from total RNA according to the manufacturer’s instructions using the Transcriptor First Strand cDNA Synthesis Kit (Roche). PCR was done on cDNA using Biolase Red DNA Polymerase and primers as mentioned in the text and Supplemental Table I. PCR to determine the expression of SRO1 in different tissues was done for 45 cycles. FLC expression was assayed using cDNA made from material collected from plants grown under short day growth conditions. rcd1-3 cDNA was made from young aerial rosettes, including the apical meristem, while wild type and sro1-1 cDNA was made from young nodes where cauline leaves were associated with several leaves. RT-PCR was done as above. Primers used to amplify FLC were as described (Wang et al., 2007).

Phenotypic Analysis of the Mutants
Root phenotypes and flowering time were analysed in wild type (Columbia), rcd1-3 and sro1-1; all other phenotypes were observed in wild type, rcd1-3, sro1-1 and rcd1-3; sro1-1. Wherever indicated in the text, significant difference between the phenotypes of the mutants and the wild type was calculated, at P < 0.01 or 0.05, by the Student’s t-test.

Plant height was measured when the plants reached maturity and the flowers of primary inflorescence had formed siliques; length of the primary inflorescence stem was measured from three independent biological replicates of each genotype. Each replicate
consisted of 10 plants of each genotype. In total, height of 30 plants of every genotype was measured and the average height and standard error calculated.

The roots of 11 days old seedlings were analysed from three independent biological replicates of each genotype. For each replicate, at least 40 seedlings of every genotype were examined. In total, 130 seedlings were analyzed. In order to determine the length of the lateral roots, the 5 longest lateral roots from each seedling was measured, for a total of at least 650 lateral roots per genotype.

Flowering time was analysed using 30 plants of each genotype in three independent biological replicates (10 plants per replicate) grown either in long day or short day conditions as described above. Both days from germination to bolting and the number of rosette leaves when the bolt length was approximately 5 cm. were recorded.

In order to determine if seeds produced by the different genotypes were normal, mature dry seeds were examined under the dissecting scope. Two independent seed stocks of each genotype with at least 100 seeds per replicate were analyzed. Based on physical appearance, seeds were placed into 3 classes: Normal, Class I and Class II (for more detail please see the Results section). In addition, mature embryos from green, fully formed seeds, were excised and observed under the microscope. DIC techniques on a Nikon Eclipse E600 microscope were used to observed mature ovules and embryonic development in wild type and rcd1-3; sro1-1 plants. Ovules were dissected from siliques collected 4 days after emasculation of mature flower buds while embryos were observed after dissection of fertilized siliques at various developmental stages. Dissected siliques were cleared with a chloral hydrate/glycerol/water solution (8:1:2 w/v/v) without prior fixation.

**Stress Response and Hormone Assays**

To assay germination, seeds of Columbia, rcd1-3 and sro1-1 were sown on MS plates containing 0.7% (w/v) agar and 3% (w/v) sucrose supplemented with NaCl, H₂O₂, or paraquat as described (Katiyar-Agarwal et al., 2006). Seed germination was defined as the emergence of cotyledons and assayed after 5 days. Three biological replicates were analyzed for each treatment and for each replicate approximately 150 seeds of each genotype were used. Evaluation of the results of these assays involved setting
germination percentage of the mock treatments for each genotype to 100% and the respective stress conditions are presented as the percentage of this value.

Osmotic stress assays were done by sowing seeds on MS medium with 0, 2, 4, or 6% glucose or mannitol (w/v), vernalized for 5 days at 4°C, and incubated as above for 4 days as described (Ahlfors et al., 2004). Germination percentage was analyzed as above.

Hormone response assays were done by plating seeds on MS + 1% sucrose supplemented with 10µM GA3 or 0.1µM BR. The seeds were vernalized for 5 days and seedlings were analyzed as indicated in the text.

**Scanning Electron Microscopy**

Two weeks old seedlings were fixed in 3% (v/v) glutaraldehyde + 2% (v/v) paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in 0.1M phosphate buffer, pH 7.2 by vacuum infiltration and then overnight at 4°C. Fixed samples were washed three times for 15 minutes each with 0.1 M potassium phosphate buffer pH 7.2. Samples were then postfixed in 1% (v/v) osmium tetroxide (Sigma-Aldrich, St. Louis, MO) for 1 hour, and dehydrated through an ethanol series 25, 50, 75, 95, and 100% (3 times). Tissues were coated with platinum and examined with a scanning electron microscope (Hitachi S-3500N) at MCIC, Ohio Agricultural Research and Development Center (OARDC), The Ohio State University, Wooster, Ohio.

**Photography**

Photographs of adult plants were taken on an Olympus digital camera (C-5500). Photographs of seedlings, flowers, seeds and embryos were taken on a Nikon Digital Sight DS-5M camera on a Nikon SMZ800 dissecting microscope. All photographs were taken with equal magnification for each plant part studied. All images of equal magnification were put into equal size canvas of same resolution to make a composite figure with Adobe® Photoshop® version 7.0.

**SUPPLEMENTAL MATERIAL**

**Supplemental Figure 1.** Phylogenetic tree of the PARP family in angiosperms.

**Supplemental Figure 2.** RCD1 and SRO1 are similar to PARP11.
**Supplemental Figure 3.** Comparison of the expression patterns of *RCD1* and *SRO1*.

**Supplemental Table I.** Primers used in this study.

**ACKNOWLEDGEMENTS**

We thank Dr. Tea Meulia and the MCIC, Ohio Agricultural Research and Development Center (OARDC), the Ohio State University, Wooster, Ohio for SEM and DIC assistance, Matteo Citarelli for generating the phylogenetic tree, the Arabidopsis Biological Resource Center for *rcd1-3* and *sro1-1* seeds, Dr. JC Jang for help with hormone experiments, Dr. Jang and Dr. Iris Meier for critical reading of the manuscript, Dr. T. Nakagawa, Shimane University, Matsue 690, Japan for the gift of the *pGWB* destination vectors, and Alyssa LaRue for assistance with media preparation, plant care, and general lab services.
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FIGURE LEGENDS

Figure 1. RCD1 and SRO1 are similar proteins. (A) Predicted amino acid sequences of the RCD1 and SRO1 proteins. SRO1 protein is 76% similar to RCD1. Asterisks, colons and periods indicate identical, similar, and semi-conserved amino acid residues, respectively. Hyphens correspond to gaps introduced to improve the alignment. The blue box marks WWE domain. The red box indicates the putative PARP catalytic domain. The insertion sites in rcd1-3 and sro1-1 are indicated by green (inverted) and blue (upright) triangles, respectively. (B) SRO1 is expressed in all plant parts tested. RT-PCR was done using primers SRO1-F and SRO1-R to amplify SRO1 and Actin-F and Actin-R to amplify the actin control gene (Supplemental Table I). (C) The T-DNA insertions in the mutant alleles disrupt gene expression. rcd1-3 and sro1-1 do not accumulate any detectable full-length transcript. RT-PCR was done using primers RCD1-F and RCD1-R and SRO1-F and SRO1-R, respectively. (D) Transcription upstream and downstream of the T-DNA insertion site is seen in sro1-1. RT-PCR upstream (upper panel; using primers SRO1-150F and SRO1-1360R) and downstream (lower panel; primers SRO1-1600F and SRO1-R) of the T-DNA insertion produces products. IN, inflorescence; S, seven day old seedlings; RL, rosette leaves; CL, cauline leaves; RT, roots.

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Table I. *rcd1-3* and *sro1-1* plants display different responses to oxidative stress.\(^{a}\)

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Col-0</th>
<th><em>rcd1-3</em></th>
<th><em>sro1-1</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>2.5 mM H(_2)O(_2)</td>
<td>83.6 ± 3.8(^{b})</td>
<td>73.5 ± 3.6</td>
<td>94.1 ± 1.9</td>
</tr>
<tr>
<td>0.25(\mu)M paraquat</td>
<td>22.5 ± 1.3</td>
<td>77.9 ± 3.3(^{c})</td>
<td>38.9 ± 2.2(^{c})</td>
</tr>
</tbody>
</table>

\(^{a}\) Seeds were plated on MS medium supplemented with indicated amounts of H\(_2\)O\(_2\) or paraquat. Seed germination was assessed as emergence of cotyledons after 5 days of light exposure. \(^{b}\) Values are the mean percentage ± SE of germinated seeds using data from three independent experiments. Values of mock were set to 100. \(^{c}\) indicates values significantly different from the wild type at P < 0.05.
Table II. rcdl-3 and sro1-1 plants display different responses to salt stress.a

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Col-0</th>
<th>rcdl-3</th>
<th>sro1-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>80 mM NaCl</td>
<td>85.8 ± 1.3 b</td>
<td>71.1 ± 7.0 c</td>
<td>92.5 ± 0.7 c</td>
</tr>
</tbody>
</table>

a Seeds were plated on MS medium supplemented with 80mM NaCl. Seed germination was assessed as emergence of cotyledons after 5 days of light exposure. b Values are the mean percentage ± SE of germinated seeds using data from three independent experiments. Values of mock were set to 100. c indicates values significantly different from the wild type at P < 0.05.
Table III. *rcd1-3* and *sro1-1* mutants are resistant to osmotic stress

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Col-0</th>
<th>rcd1-3</th>
<th>sro1-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>2% glucose</td>
<td>92.9 ± 0.6 b</td>
<td>91.1 ± 0.4</td>
<td>93.8 ± 1.9</td>
</tr>
<tr>
<td>4% glucose</td>
<td>78.7 ± 4.1</td>
<td>80.1 ± 1.8</td>
<td>81.7 ± 0.6</td>
</tr>
<tr>
<td>6% glucose</td>
<td>36.5 ± 3.5</td>
<td>54.7 ± 5.6 c</td>
<td>64.5 ± 2.0 c</td>
</tr>
<tr>
<td>2% mannitol</td>
<td>97.1 ± 1.2</td>
<td>95.4 ± 1.8</td>
<td>99.2 ± 0.3</td>
</tr>
<tr>
<td>4% mannitol</td>
<td>87.7 ± 1.2</td>
<td>96.1 ± 1.6 c</td>
<td>96.1 ± 0.8 c</td>
</tr>
<tr>
<td>6% mannitol</td>
<td>58.2 ± 2.2</td>
<td>73.9 ± 0.6 c</td>
<td>93.0 ± 0.5 c</td>
</tr>
</tbody>
</table>

*a* Seeds were plated on MS medium supplemented with indicated amounts of glucose or mannitol. Seed germination was assessed as emergence of cotyledons after 4 days of light exposure. *b*Values are the mean percentage ± SE of germinated seeds using data from three independent experiments. Values of mock were set to 100. *c*indicates values significantly different from the wild type at P < 0.05.
Table IV. RCD1 and SRO1 control plant height.

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Height (in cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col-0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>47.13 ± 0.84&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>sro1-1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>45.4 ± 0.81</td>
</tr>
<tr>
<td>rcd1-3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23.2 ± 0.58&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>rcd1-3;sro1-1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.35 ± 0.49&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>35S::RCD1;rcd1-3 (5)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>43.6 ± 1.04</td>
</tr>
<tr>
<td>35S::RCD1;rcd1-3 (7)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>43.4 ± 0.97</td>
</tr>
<tr>
<td>35S::RCD1;rcd1-3 (8)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>46.0 ± 0.93</td>
</tr>
<tr>
<td>Col-0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>42.7 ± 1.01</td>
</tr>
<tr>
<td>rcd1-3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>22.16 ± 0.57&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>rcd1-3;+/+&lt;sup&gt;c&lt;/sup&gt;</td>
<td>21.67 ± 0.54</td>
</tr>
<tr>
<td>rcd1-3;sro1-1/+&lt;sup&gt;c&lt;/sup&gt;</td>
<td>14.94 ± 0.68&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a, b, c</sup> Plants grown in the respective identical conditions. (5), (7) and (8) represent three independent transgenic lines. <sup>d</sup>Values are the mean ± SE. <sup>e</sup>indicate that values are significantly different from the wild type at P < 0.01.
Table V. *rcdl-3; sro1-1* seeds are abnormal.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Wild type</th>
<th>Class I&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Class II&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col-0</td>
<td>83.8 ± 10.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.5 ± 6.0</td>
<td>5.16 ± 4.2</td>
</tr>
<tr>
<td><em>rcdl-3</em></td>
<td>82.6 ± 5.25</td>
<td>11.9 ± 5.37</td>
<td>5.4 ± 0.19</td>
</tr>
<tr>
<td><em>sro1-1</em></td>
<td>75.2 ± 1.9</td>
<td>18.5 ± 1.04</td>
<td>6.15 ± 2.95</td>
</tr>
<tr>
<td><em>rcdl-3; sro1-1</em></td>
<td>7.4 ± 2.6</td>
<td>59.6 ± 1.85</td>
<td>33.0 ± 0.75</td>
</tr>
</tbody>
</table>

<sup>a</sup> Classes as defined in text. <sup>b</sup> Values are the mean percentage ± SE from two independent seed stocks.
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