

Arabidopsis RADICAL-INDUCED CELL DEATH1 Belongs to the WWE Protein–Protein Interaction Domain Protein Family and Modulates Absciscic Acid, Ethylene, and Methyl Jasmonate Responses

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Experiments with several *Arabidopsis thaliana* mutants have revealed a web of interactions between hormonal signaling. Here, we show that the *Arabidopsis* mutant *radical-induced cell death1* (*rcd1*), although hypersensitive to apoplastic superoxide and ozone, is more resistant to chloroplastic superoxide formation, exhibits reduced sensitivity to absciscic acid, ethylene, and methyl jasmonate, and has altered expression of several hormonally regulated genes. Furthermore, *rcd1* has higher stomatal conductance than the wild type. The *rcd1-1* mutation was mapped to the gene At1g32230 where it disrupts an intron splice site resulting in a truncated protein. RCD1 belongs to the (ADP-ribosyl)transferase domain-containing subfamily of the WWE protein–protein interaction domain protein family. The results suggest that RCD1 could act as an integrative node in hormonal signaling and in the regulation of several stress-responsive genes.

INTRODUCTION

The production of reactive oxygen species (ROS) is a common feature of plant responses to abiotic and biotic stress. ROS production triggers a network of signaling events leading to several outputs, including stress tolerance, acclimation, and cell death (Dat et al., 2000; Mittler, 2002; Vranová et al., 2002). ROS are also involved in the regulation of the closure of stomata, gravitropism in the root tip, and root branching (Joo et al., 2001; Foreman et al., 2003; Kwak et al., 2003). In all these processes, increased ROS production is functionally coupled to the action of

plant hormones such as ethylene (ET), salicylic acid (SA), jasmonic acid (JA), and absciscic acid (ABA).

The use of *Arabidopsis thaliana* mutants has been instrumental in the dissection of hormone signaling networks and their interactions. For example, upon ozone (O₃) exposure, the *radical-induced cell death1* (*rcd1*) mutant accumulates SA and ET, which together promote continuous ROS production and cell death, whereas methyl jasmonate (MeJA) acts antagonistically to ET inhibiting lesion spread (Overmyer et al., 2000, 2003; Langebartels and Kangasjärvi, 2004). At the transcriptional level, these hormones act both synergistically and antagonistically in regulating stress-induced gene expression (Tuominen et al., 2004). JA and ET together are required for induction of *PDF1.2* expression, which is inhibited by SA. Conversely, mutant phenotypes indicate that *PR1* expression is induced by SA and inhibited by JA (Turner et al., 2002). In the *hypersensitive response-like lesions1* mutant, induction of *PR1* expression requires both SA and ET, and induction of *PDF1.2* expression requires all three hormones SA, ET, and JA (Devadas et al., 2002), indicating further levels of complexity in hormone interactions. Hence, it is likely that the timing, location, and levels of the various hormones determine the interaction (antagonistic or synergistic) and output responses.

Hormones regulate stress-induced gene expression via modulation of each other's synthesis and signaling. For example, ET production increases the biosynthesis of ABA (Grossmann, 2003). The balance between these two hormones is maintained through subsequent inhibition of ET biosynthesis by ABA and by

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mutually antagonistic interactions between ABA and ET signaling (Fedoroff, 2002). This antagonistic interaction between ABA and ET was elucidated with the ET mutants *ein2* and *ctr1*, which act as a suppressor and an enhancer, respectively, of the ABA-insensitive *abi1* (Beaudoin et al., 2000; Ghassemian et al., 2000). Accordingly, *ein2* overproduces ABA (Ghassemian et al., 2000). Several ABA mutants together with constitutive ET signaling mutants also appear to be insensitive to glucose, and ABA has been established as a regulator of glucose signaling through double mutant analysis. ET also modulates glucose signaling through the regulation of ABA (León and Sheen, 2003).

ABA is involved in responses to several abiotic stresses and is a well-known regulator of stomatal closure. In stomata, ABA induces ROS production (Pei et al., 2000). Recently, direct genetic evidence for the requirement of ROS in ABA-induced stomatal closure was shown with mutants of the plasma membrane NADPH oxidase catalytic subunits *atrbohF* and *atrbohD* *atrbohF*, which exhibited impaired ABA-induced stomatal closing (Kwak et al., 2003).

As a potential interactor between hormones and ROS formation, the O_3 -sensitive *rcd1* mutant (Overmyer et al., 2000) offers a tool to study hormonal interactions in relation to ROS production addressing the importance of the subcellular site of ROS action. We report here the map-based identification of *RCD1*, followed by genetic and physiological analyses for the elucidation of processes in which the RCD1 protein is involved. *RCD1* encodes a protein of unknown function that belongs to a family of proteins bearing WWE protein-protein interaction domains. RCD1 was found to be involved in a subset of responses to several plant hormones, including ABA, ET, and MeJA.

RESULTS

rcd1 Is Sensitive to Apoplastic ROS but Tolerant to Chloroplastic ROS

Mutant *rcd1* plants were initially isolated on the basis of their enhanced sensitivity to extracellular ROS generated by O_3 exposure. In addition to O_3 sensitivity, which has been described previously (Overmyer et al., 2000), *rcd1* plants also have several other distinctive phenotypes, including smaller, more erect rosettes, altered leaf shape, and earlier flowering (Figure 1A), which all cosegregate with O_3 sensitivity (Table 1). To differentiate whether the codominant ozone-sensitive phenotype of *rcd1* was a result of haplo-insufficiency or a gain of function, triploid *rcd1/RCD1/RCD1* plants were produced and tested for ozone sensitivity. The O_3 sensitivity of the triploid plants was similar to heterozygous *rcd1/RCD1* plants (data not shown), implying that *rcd1* confers O_3 sensitivity by a gain of function. Tables 1 and 2 indicate that both the morphological and ROS sensitivity phenotypes are conferred either by two very tightly linked genes or by the same gene. Because *rcd1* is sensitive to apoplastic ROS (Overmyer et al., 2000), we tested whether *rcd1* is also sensitive to ROS produced inside the cell with plants grown on MS plates containing 1 μ M herbicide paraquat, which causes generation of excess ROS within the chloroplast (Mehler, 1951). Unexpectedly, *rcd1* was more tolerant to paraquat than Columbia-0 (Col-0); it

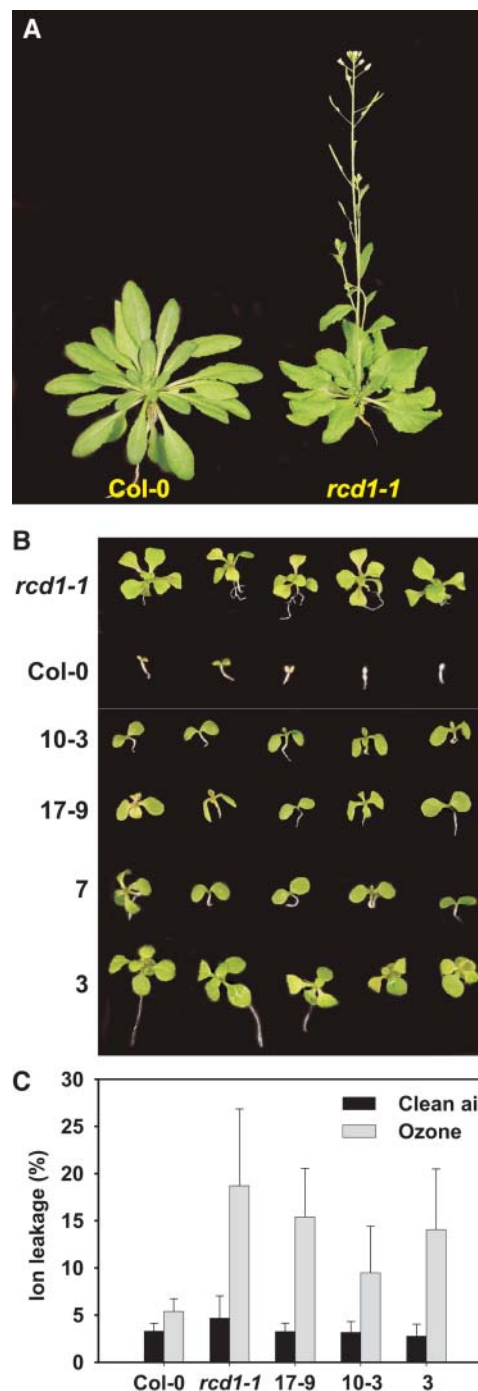


Figure 1. *rcd1-1* Phenotypes.

(A) Habitus of 4-week-old Col-0 and homozygous *rcd1-1/rcd1-1* plants. **(B)** Paraquat tolerance of 2-week-old seedlings grown in 1.0 μ M paraquat. A single copy (10-3 and 17-9) or multiple copies (7 and 3) of the *rcd1-1* cDNA transgene confer tolerance to paraquat when expressed under the CaMV 35S promoter in the paraquat sensitive wild-type Col-0. **(C)** Ozone sensitivity of 3-week-old plants. Cell death (measured as ion leakage) after a 4-h 250 nL L⁻¹ O_3 exposure in the Col-0 wild type, mutant *rcd1-1*, and the transgenic lines 17-9, 10-3, and 3 expressing the *rcd1-1* cDNA in Col-0.

Table 1. The Segregation of *rcd1-1* Habitus and O₃ Sensitivity Phenotypes

	Col-0 Habitus		<i>rcd1</i> Habitus		Total		Model
	Obs	Exp	Obs	Exp	Obs	Exp	
O ₃ sensitive	42	0	145	189.5	187	189.5	1
Intermediate	358	379.0	28	0	389	379.0	2
O ₃ tolerant	177	189.5	8	0	182	189.5	1
Total	577	568.5	181	189.5	758		
Model	3		1				

The F2 progeny of an *rcd1-1* × Col-0 backcross were scored for the *rcd1* habitus phenotype and O₃ sensitivity. 1:2:1 Model $\chi^2 = 0.593668$ (df = 3), 3:1 model $\chi^2 = 0.508355$ (df = 1). Data are the pooled results of four independent O₃ exposures from two independent crosses. Representative individual lines (five lines of each class) that deviate from the model (such as O₃ sensitive plants with a Col-0 habitus and intermediate sensitive or tolerant plants with an *rcd1* habitus) were retested as populations of F3 progeny. Retesting revealed that in these inconsistent lines, F3 phenotypes were consistent with the model and had been mis-scored in the F2, likely because of physiological variations. All *rcd1* habitus plants in the F2 had 100% O₃ sensitive *rcd1* habitus progeny in the F3. Lines that were scored in the F2 as O₃ sensitive Col-0 plants retested in the F3 as either 100% O₃ tolerant Col-0 habitus plants or segregated for both O₃ sensitivity and habitus phenotypes.

was able to germinate and develop true leaves, whereas Col-0 germinated but quickly bleached and died thereafter (Figure 1B). Also surprisingly, whereas the sensitivity to apoplastic ROS (i.e., O₃ or exogenous superoxide) segregated as a codominant trait (Overmyer et al., 2000; Table 1), the tolerance to paraquat and the mutant habitus morphology cosegregated as simple recessive traits (Table 2). These unique genetics are characteristic of not only the *rcd1-1* allele. A second *rcd1* allele, *rcd1-2*, isolated in a screen for paraquat tolerant mutants, is also sensitive to O₃ (Fujibe et al., 2004).

rcd1 Maps to the Gene At1g32230

Linkage was initially established on chromosome 1 (Overmyer et al., 2000). Genetic mapping was used (Figure 2A; Methods) to define *rcd1-1* to an interval on the BACs F3C3 and F27G20 containing seven open reading frames, six of which matched the published Arabidopsis genome sequence perfectly. The gene At1g32230 harbored a single C-to-T transition, typical to ethyl methanesulfonate mutagenesis, on the antisense strand resulting in a G-to-A transition at the GT splice site of the third exon-intron junction. The nucleotide sequence of At1g32230 from three nonallelic O₃-sensitive mutants isolated in the same screen was identical to the published Col-0 genome sequence, indicating that the base change resulted from the ethyl methanesulfonate mutagenesis and was not present in the original seed population.

Complementation tests confirmed that the mutant phenotype is conferred by the mutation observed. Expression of the *rcd1-1* cDNA under the 35S promoter of *Cauliflower mosaic virus* (CaMV) in Col-0 increased the tolerance of the transgenic lines to paraquat (Figure 1B) and sensitivity to O₃ (Figure 1C). The use of CaMV 35S promoter to overexpress the mutant cDNA in

Col-0 resulted in significantly higher transcript abundance for the mutant *rcd1-1* cDNA than the wild-type *RCD1* cDNA (data not shown), which is also clearly visible as a difference in the paraquat resistance between the transgenic plants that contain one copy (lines 10-3 and 17-9) or two copies (lines 7 and 3) of the 35S-regulated *rcd1-1* cDNA (Figure 1B). Thus, overexpression of the mutant protein results in a dominant-like complementation of a recessive paraquat resistance because of the higher abundance of the mutant protein in the transgenic complementation lines. The F1 progeny from a cross between *rcd1-1* and *rcd1-2* also bears both paraquat and O₃ phenotypes and leaf morphology of *rcd1* (Fujibe et al., 2004; data not shown), indicating a lack of genetic complementation of both phenotypes in these two independently isolated mutants. These results confirm that the phenotypes are associated with the mutation in At1g32230.

RCD1 Belongs to a Subfamily of WWE Domain-Containing Proteins

The *RCD1* protein coding sequence matches a RIKEN Arabidopsis full-length cDNA sequence (AY142655) except for an insertion of three bases (CAG) resulting in an extra glutamine residue at Q475. RCD1 is identical to CEO1, which was identified as a Col-0 cDNA that complements an oxidative stress-sensitive yeast strain (Belles-Boix et al., 2000). It is also 95% identical with a Landsberg *erecta* (Ler) protein published as ATP8 (Lin and Heaton, 2001), which was identified in a yeast two-hybrid screen using turnip crinkle virus movement protein as bait. The difference between RCD1 and ATP8 sequences is mostly attributable to a deletion in *Ler* resulting in a 17-amino acid shorter protein.

The predicted RCD1 protein has a molecular weight of 66 kD and a pI of 6.46. RCD1 is presumed to be targeted to the nucleus based on two potential nuclear localization signals, GKRRKRA at position 18 and KRRRL at position 54. Database searches identified an Arabidopsis paralog (At2g35510) of RCD1 with 76% similarity, named as SRO1 (for SIMILAR TO RCD ONE1). Other related genes are a rice ortholog (*Oryza sativa*; unknown protein AC35157.2) with 53% similarity to RCD1 and four shorter Arabidopsis proteins with 43 to 49% similarity to RCD1 (At1g23550, At1g70440, At3g47720, and At5g62520, which will be referred to as SRO2, SRO3, SRO4, and SRO5, respectively).

Aravind (2001) classified RCD1 in a novel subfamily of proteins, involved in ubiquitin and ADP-ribose conjugation systems, that contain a conserved globular domain called the WWE domain (PF02825; IPR004170) predicted to mediate specific protein-protein interactions. RCD1 (F3C3.1_At_10801372) was the only

Table 2. The Segregation of *rcd1-1* Habitus and Paraquat Tolerance Phenotypes

	Col-0 Habitus	<i>rcd1</i> Habitus	Total	Model
Paraquat sensitive	76	0	76	3
Paraquat tolerant	0	24	24	1
Total	76	24	100	
Model	3	1		

The F2 progeny of an *rcd1-1* × Col-0 backcross were scored for the *rcd1* habitus phenotype and paraquat tolerance. See Table 1 legend for details.

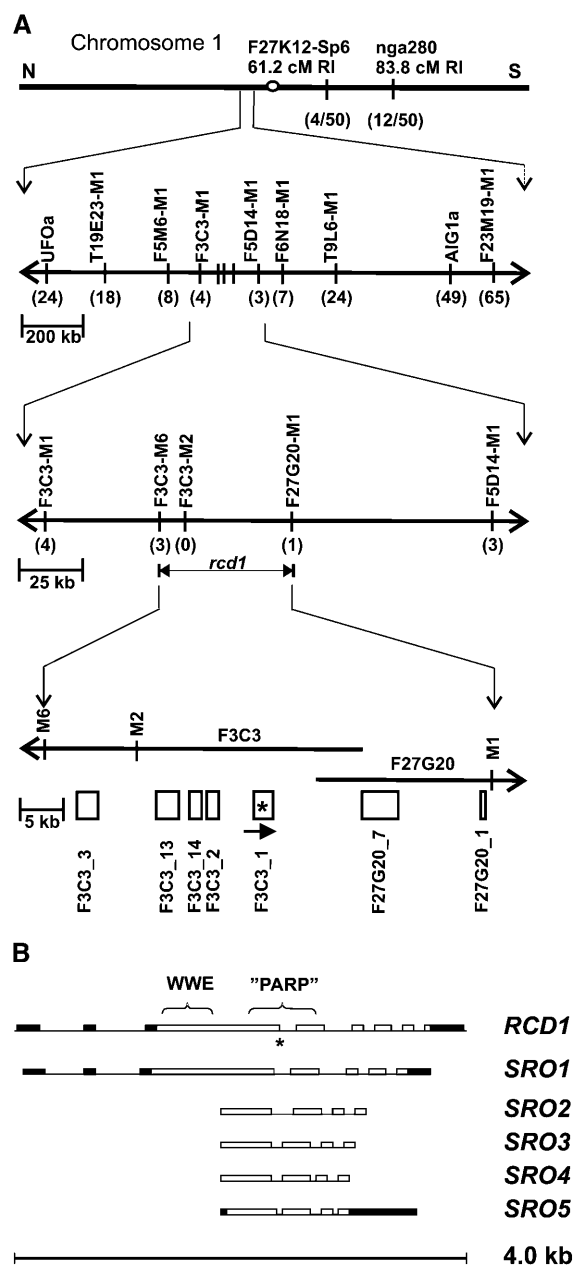


Figure 2. *RCD1* Map Position and Arabidopsis *SIMILAR TO RCD1* Gene Family.

(A) Positional cloning of *rcd1*. Flanking (UFOa and F23M19-M1) and additional markers in between were used to identify recombinant offspring from the mapping cross between Ler (*RCD1/RCD1*) and Col (*rcd1/rcd1*).

(B) Structure of *RCD1*-like family gene transcripts. Black boxes, untranslated regions; gray boxes, exons. The position of the *rcd1-1* mutation at the end of exon III (and within the PARP-like domain in the encoded proteins) is indicated with an asterisk. Proteins encoded by *SIMILAR TO RCD2* (*SRO2*) to *SRO5* do not contain the WWE domain.

plant protein that was originally used by Aravind (2001) to define the WWE domain. The WWE domain is present in *RCD1*, *SRO1*, and the rice ortholog AC35157.2 but is missing from the shorter (*SRO2* to *SRO5*) proteins. *RCD1* and the *SRO* proteins also contain a region (amino acids 317 to 416 of *RCD1*) similar to the catalytic domain of poly(ADP-ribose)polymerase (PARP signature, pfam00644) (Figure 2B.) The same domain also has 46 to 47% similarity to three polyADP-ribose metabolism enzymes of *Caenorhabditis elegans* (PME-1, PME-5, and PME-6; Gagnon et al., 2002). PSI-BLAST searches identified from different organisms several ADP-ribose-related proteins that have similarity with this region. PSI-BLAST analyses also identified nine mammalian proteins of unknown function that have a low, 15 to 20% overall amino acid identity with *RCD1*, but all have both WWE and PARP domains with similar domain architecture as in *RCD1* (data not shown).

RCD1 Expression and Intron Splicing in the Wild Type and *rcd1-1*

RT-PCR indicated that in the wild-type Col-0, *RCD1* intron splicing was in agreement with the predicted At1g32230 structure and the published *CEO1* and RIKEN full-length cDNAs (data not shown). In *rcd1-1*, two types of transcripts were found by RT-PCR amplification and sequence analysis. The 2.7-kb transcript represents mRNA where the next (GT) in intron III downstream of the mutated splice acceptor site has been used in the splicing of intron III. This misspliced *rcd1-1* mRNA is only 11 bp longer than the wild-type mRNA. In *rcd1-1*, a shorter, 1.4-kb transcript is present at approximately the same expression level as the 2.7-kb transcript. In the 1.4-kb transcript, intron splicing has occurred from the 5' end of exon IV into exon III so that exon III is completely missing (data not shown). Both misspliced *rcd1-1* transcript types result in loss of reading frame and a premature stop codon. Similarly, *rcd1-2* (Fujibe et al., 2004) has a missense mutation causing a premature stop codon just a few amino acids upstream of the *rcd1-1* mutation (K. Yamamoto, personal communication) creating virtually the same truncated protein as *rcd1-1*.

In the wild-type Col-0, *RCD1* had a constitutive level of expression in stems, leaves, buds, and young flowers, whereas in old flowers, siliques and roots transcript levels were low (Figure 3A). In *rcd1-1*, expression of the gene was slightly elevated compared with the wild type, and in contrast with Col-0, was also expressed in old flowers and roots (Figure 3B). In siliques of *rcd1-1*, only the shorter transcript was present in low abundance. Exposure to O_3 (250 nL L⁻¹) for 7 h increased *RCD1* mRNA levels in Col-0 only approximately twofold to threefold (Figure 3C), whereas the *CHIB* and *PR5*, which are known to be O_3 inducible, were induced approximately fourfold to sevenfold.

ET- and ABA-Regulated Genes Have Altered Expression in *rcd1-1*

Yeast two-hybrid analysis with the *RCD1* (*CEO1*) protein identified several transcription factors, for example DREB2A, as *RCD1* interacting partners (Belles-Boix et al., 2000), which suggests that *RCD1* might be involved in regulating gene expression by affecting the function or activity of these

transcription factors. Microarray analysis was used to identify genes regulated by RCD1 under normal growth conditions. Of the 6500 genes analyzed, nine had consistent and statistically significantly higher, and two lower basal mRNA levels in *rcd1-1* in all hybridizations performed (Table 3). Two of the upregulated genes, encoding for 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase and alternative oxidase (AOX1A), are regulated by ET (Simons et al., 1999; Petruzzelli et al., 2000), and three are related to carbohydrate metabolism. The two downregulated genes (*RD29A/LTI78* and *KIN2*) are involved in cold and dehydration stress and are regulated by ABA (Nordin et al., 1993; Foster and Chua, 1999). For an independent evaluation and to expand the microarray data, a dot blot macroarray containing 92 defense- and stress-regulated genes was hybridized with ³³P-labeled cDNA probe. Two of the upregulated or downregulated genes in the microarray (*AOX1A* and *RD29A/LTI78*) were also present on the macroarray and behaved consistently in both arrays (Table 3). According to the macroarrays, a larger group of cold- and ABA-regulated genes had reduced basal transcript levels in *rcd1-1*. When the response of *RAB18* to ABA was analyzed with RNA gel blot analysis, the induction of the gene by ABA was considerably lower in *rcd1-1* than in Col-0 (Figure 4).

rcd1-1 Has Higher Stomatal Conductance

Impaired expression of ABA-related genes prompted us to analyze the stomatal conductance of *rcd1-1*. The ABA-insensitive *abi2* was used as a reference. In clean air, *rcd1-1* had ~40% higher stomatal conductance than Col-0 (Figure 5A) but lower than in *abi2* (Figure 5B, 0 h). O₃ induced differential decrease in stomatal conductance in the accessions analyzed (Figure 5B); 2 h of O₃ decreased conductance in Col-0 by 40% but only by 20% in *rcd1-1*. After 4 h, *rcd1-1* had conductance similar to Col-0, whereas *abi2* had only the level present in *Ler* preceding exposure (Figure 5B). The higher stomatal conductance in *abi2* and *rcd1-1* also had an effect on transpiration; the weight loss of detached leaves was considerably higher in *abi2* and slightly but statistically significantly higher in *rcd1-1* than in the corresponding wild types (Figure 5C).

Cold Acclimation Is Not Affected in *rcd1-1*

Because *rcd1-1* had a lower basal level and exhibited impaired induction of some cold- and ABA-responsive genes, we investigated the cold acclimation capability of the mutant. Frost

Table 3. A Microarray Containing ~6500 Different Genes Was Hybridized with RNA Isolated from Nonstressed Col-0 and the *rcd1-1* Mutant

AGI ID ^a	Annotation	Ratio	<i>t</i> Test P Value
Microarray Data			
At2g21640	Expressed protein	9.4	1.84E-07
At5g43450	ACC oxidase	6.7	1.16E-07
At3g22370	AOX1A, mitochondrial	4.4	5.61E-12
At3g08590	2,3-Bisphosphoglycerate-independent phosphoglycerate mutase, putative	3.2	1.25E-05
At3g45730	Expressed protein, targeted to chloroplasts	3.1	4.00E-05
At2g36460	Fructose-biphosphate aldolase, putative	2.6	2.71E-08
At1g14720	Xyloglucan endotransglycosylase (XTR2)	2.6	8.63E-07
At5g66052	Expressed membrane protein	2.5	9.34E-05
At3g27060	Ribonucleoside-diphosphate reductase small chain, putative	2.4	0.000312
At5g15970	Stress-induced protein (KIN2)/cold-regulated protein (COR6.6)	0.5	0.00291
At5g52310	Low temperature-induced protein 78 (LTI78)/desiccation-responsive protein 29A (RD29A)	0.5	0.00147
Macroarray Data			
		Repeat 1	Repeat 2
At3g22370	AOX1A, mitochondrial	9.5	14.5
At1g20440	Dehydrin (COR47)	1.3	0.3
At3g50970	Dehydrin Xero2 (LTI30)	0.4	0.4
At1g20450	Dehydrin erd10 (LTI29)	0.4	0.4
At5g66400	Dehydrin (RAB18)	0.3	0.5
At5g52310	LTI78/RD29A	0.2	0.3

The ratio is calculated by dividing the normalized *rcd1-1* channel intensity with the Col-0 channel intensity. The ratio is the mean value of 18 measurements (3 biological repeats*2 dyeswap technical repeats*3 triplicated spots). A one-sample Student's *t* test is calculated to test whether the mean normalized expression level for the gene is statistically different from 1.0. Macroarray data shows expression of selected stress- and defense-related genes in wild-type Col-0 and the *rcd1-1* mutant. The values depict the ratio of *rcd1-1*/Col-0 mRNA abundance from two biological repeats. The data from the phosphor imager analysis of the macroarray was first normalized to the mean of mRNA abundance of actin genes *ACT2* (At3g18780.1) and *ACT8* (At1g49240.1), which were shown to be constitutively expressed by RNA gel blots. A complete list of the genes used can be seen at <http://www.biocenter.helsinki.fi/bi/koivu/Arabidopsis/arrays.html>.

^aArabidopsis Genome Initiative identifier.

tolerance of both nonacclimated plants and plants exposed for 2 d to exogenous ABA or to 4°C was tested by controlled freezing of excised leaves. When ion leakage was measured as an indicator of freezing-induced tissue damage, a similar increase at −6°C in both nonacclimated *rcd1-1* and Col-0 indicated that the mutation in *RCD1* does not affect nonacclimated freezing tolerance (Figure 6). Acclimating plants with ABA (Figure 6A) or at 4°C (Figure 6B) before exposure to freezing temperatures equally decreased freezing-induced tissue damage in both genotypes, indicating that the capability to cold acclimate is not significantly impaired in *rcd1-1*. However, ABA- and cold-acclimated as well as nonacclimated *rcd1-1* at −6 and −8°C showed somewhat higher electrolyte leakage than Col-0, which might indicate a slightly increased freezing sensitivity for *rcd1-1*.

RCD1 Is Involved in ET and MeJA Responses

Because stress hormone-related gene expression showed alterations in *rcd1-1* (Table 3; Overmyer et al., 2000), we characterized its sensitivity to ET, JA, and SA. In the triple response

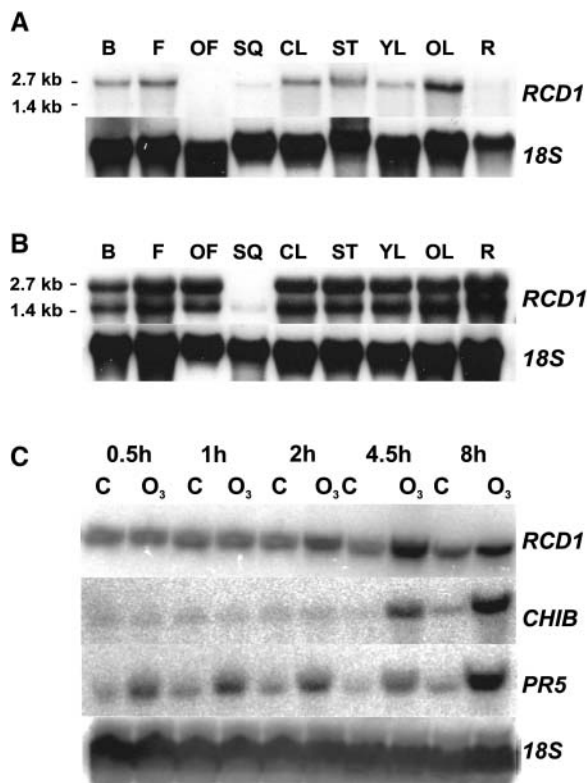


Figure 3. Expression of *RCD1* and Defense Response Marker Genes.

(A) and (B) RNA gel blot analysis of *RCD1* expression in various tissues of Col-0 (A) and *rcd1-1* (B). B, buds; F, flowers; OF, old flowers; SQ, siliques; CL, cauline leaves; ST, stem; YL, young leaves; OL, old leaves; R, root. (C) Expression of *RCD1*, *PR5*, and *CHIB* in Col-0 exposed to 250 nL L^{−1} O₃ for 7 h. Samples were harvested after 0.5, 1, 2, 4.5, and 8 h. C, clean air control; O₃, ozone. The blots were reprobed with 18S rDNA to equalize for loading differences.

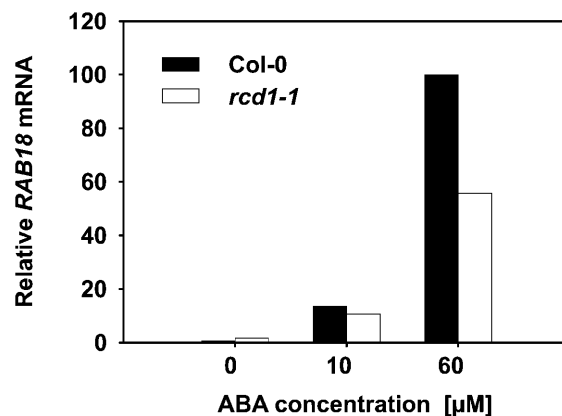


Figure 4. Expression of *RAB18* in ABA-Treated Col-0 and *rcd1-1*.

RNA gel blot hybridization analysis of *RAB18* expression in rosette leaves treated with 0, 10, and 60 μM ABA. The signal intensity was normalized with 18S rDNA for loading differences.

assay, hypocotyl length in *rcd1-1* did not differ from Col-0 wild type (Figure 7A). Thus, in this generally accepted marker for reduced ET sensitivity, *rcd1* cannot be regarded as ET insensitive. However, exogenously applied ET precursor ACC induced the ET-responsive gene *CHIB* in *rcd1-1* to the same extent as in the ET-insensitive *ein2*, and when in Col-0 considerably higher *CHIB* transcript accumulation was evident (Figure 7B). The induction of ET biosynthetic genes (ACC oxidase, *ACO1*, and ACC synthase, *AT-ACS6*) and ET evolution were also increased in *rcd1-1* (Table 3; Overmyer et al., 2000), which is consistent with loss of feedback regulation of the biosynthesis because of insensitivity to the hormone.

The responsiveness of *rcd1-1* plants to JA was also altered. We have previously shown that *rcd1-1* can sense MeJA normally in an assay for inhibition of root growth (Overmyer et al., 2000). However, in MeJA-treated *rcd1-1*, expression of *VSP1* was lower than in Col-0 but not as low as in the MeJA-insensitive mutant *jar1* (Figure 7C). Interestingly, induction of *VSP1* expression by MeJA in *rcd1-1* was comparable to that in *ein2*. Induction of *PR-1* expression by SA was not affected in *rcd1-1* (data not shown).

rcd1-1 Exhibits Moderate Insensitivity to Glucose

ABA, ET, and glucose signaling are known to interact (León and Sheen, 2003). Because *rcd1-1* has reduced sensitivity to ET and ABA, its response to glucose was tested. Compared with Col-0, *rcd1-1* exhibited moderate glucose insensitivity; it was able to germinate normally and develop green cotyledons on 2% glucose when Col-0 germinated but development of its cotyledons was repressed (Figure 8). The ET-insensitive *ein2* displayed a glucose-hypersensitive phenotype. On 4% glucose, the development of *rcd1-1* seedlings was deferred, although less than in Col-0, and 6% glucose inhibited the development of both Col-0 and *rcd1-1* seedlings in a similar manner (data not shown). Interestingly, the *rcd1-1* mutation partially suppressed the glucose-hypersensitive phenotype of *ein2* in the *rcd1 ein2* double mutant.

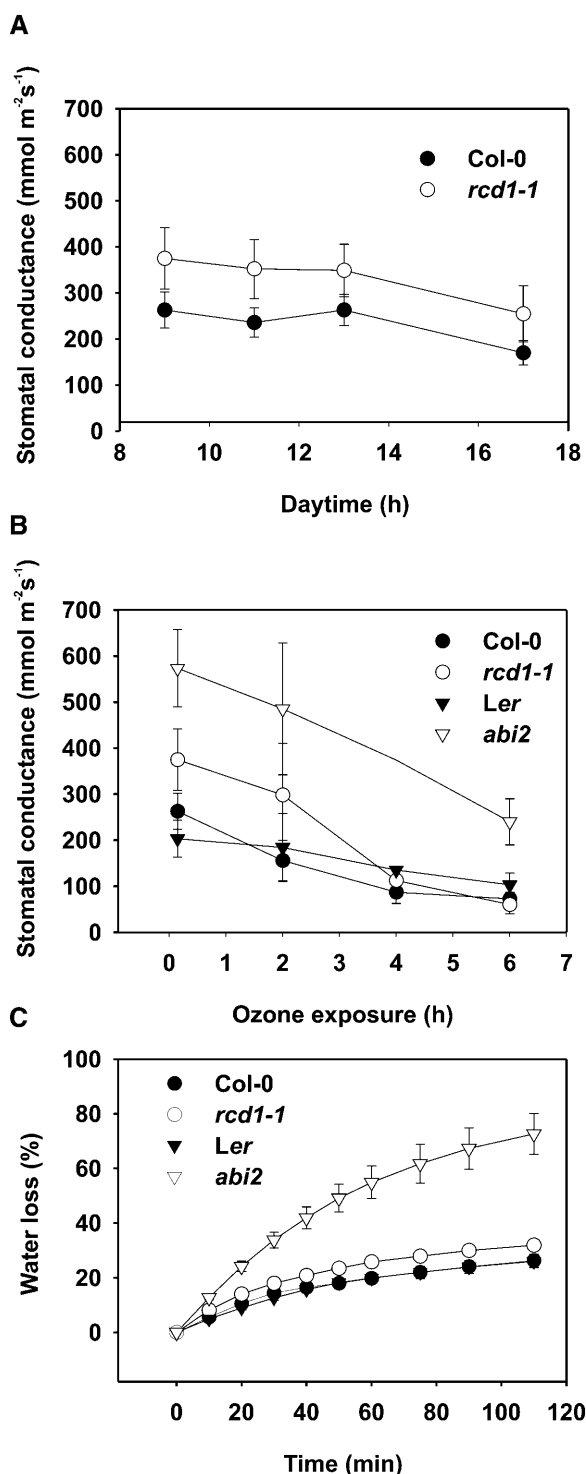


Figure 5. *rcd1-1* Has Higher Stomatal Conductance.

(A) Stomatal conductance of 24-d-old *rcd1-1* and Col-0 wild type.
(B) Stomatal conductance of 24-d-old *rcd1-1*, *abi2*, and corresponding wild-type plants during a 6-h O₃ treatment of 250 nL L⁻¹ from 9:00 to 15:00 h. The error bars in **(A)** and **(B)** indicate standard deviation of two to three leaves from each of five plants.

DISCUSSION

Specificity of ROS Generation in the Apoplast and Chloroplast

Plants respond to different ROS by activation of specific sets of genes, depending on the type of ROS, and/or their subcellular site of production. For example, in the Arabidopsis mutant *flu*, singlet oxygen generated in the plastid activated different sets of genes than O₂^{-•} and H₂O₂ (op den Camp et al., 2003). Although O₃ and paraquat produce the same ROS (O₂^{-•} and H₂O₂), *rcd1* is sensitive to O₃ but resistant to paraquat. The differential sensitivity most likely results from the different subcellular location of ROS production; paraquat acts in the chloroplast (Mehler, 1951), whereas O₃ generates O₂^{-•} and H₂O₂ within the apoplast (Kangasjärvi et al., 1994).

Evidence from several experiments suggests the presence of an apoplastic superoxide dismutase (SOD) responsible for the dismutation of O₂^{-•} into H₂O₂ in the cell wall (Ogawa et al., 1997; Schinkel et al., 1998). The accumulation of O₂^{-•} in the apoplast of *rcd1-1* (Overmyer et al., 2000) could be an indication of a lower apoplastic SOD activity, which could be compensated by higher expression of SODs and ascorbate peroxidases (APX) delivered to other subcellular compartments (e.g., to the chloroplast). This kind of compensation maintaining stable total SOD activity has been seen in SOD antisense and overexpression lines (Kliebenstein et al., 1998) and would explain the increased paraquat tolerance in the *rcd1* mutants. Accordingly, *rcd1* has higher mRNA levels for the chloroplastic *FeSOD1* (Overmyer et al., 2000) and for the thylakoid-bound *APX* (Fujibe et al., 2004). Furthermore, UV-B irradiation-induced oxidative stress increased the transcript levels of the chloroplastic *FeSOD*, *CuZnSOD*, and *APX* in *rcd1-2* (Fujibe et al., 2004).

Is *rcd1* an Ozone Sensitive or Hormone Mutant?

The higher stomatal conductance in *rcd1* raises the question whether its sensitivity to O₃ could simply result from a higher influx of O₃ and not necessarily from a higher sensitivity to O₃ per se. Xanthine/xanthine oxidase (X/XO), a stomata-independent extracellular superoxide generating system, triggered about the same level of primary cell death in both *rcd1-1* and Col-0 (Overmyer et al., 2000). After infiltration, X/XO produces a strong burst of O₂^{-•} that can cause direct damage. Accordingly, in the X/XO-infiltrated plants, the primary damage, presumably a direct result of the apoplastic ROS production from X/XO, was more or less the same in both *rcd1-1* and Col-0. However, in Col-0, the ROS-dependent propagation of lesions did not take place, which

(C) Water loss from detached leaves of *rcd1-1*, *abi2*, and corresponding wild types. Weight of leaves cut from four 21-d-old plants were monitored for 2 h. The error bars in **(C)** (shown when wider than the symbol) indicate standard deviation for the four plants from which the leaves were cut and weighed together. Water loss from *rcd1-1* differs significantly from the Col-0 wild type (analysis of variance, $P < 0.0005$).

suggests a role for RCD1 in the regulation of ROS-related lesion propagation.

It has been shown that the lesion propagation in O_3 damage is under hormonal control (Overmyer et al., 2000; Rao et al., 2000, 2002; Moeder et al., 2002; Kanna et al., 2003). Both jasmonate-insensitive (*jar1* and *coi1*) and ET-overproducing mutants (*eto1* and *eto2*) are O_3 sensitive as a result of lesion propagation, whereas the ET- or SA-insensitive mutants (*etr1*, *ein2*, *npr1*, and *NahG*) are O_3 tolerant. Furthermore, the mutants and accessions that have first been described as O_3 sensitive have also turned out to be partially JA insensitive (*oji1*, *rcd1*, and Cvi-0) or ET overproducers (*rcd1*) (Overmyer et al., 2000; Rao et al., 2000; Kanna et al., 2003). Moreover, the endogenous ROS production triggered by O_3 is ET dependent (Overmyer et al., 2000; Moeder et al., 2002; Wohlgemuth et al., 2002; Kanna et al., 2003). Thus, it is conceivable that in several instances O_3 sensitivity is a result of altered interactions between hormonal signaling pathways, which regulate the propagation of cell death through endogenous ROS production (Overmyer et al., 2003). Accordingly, O_3

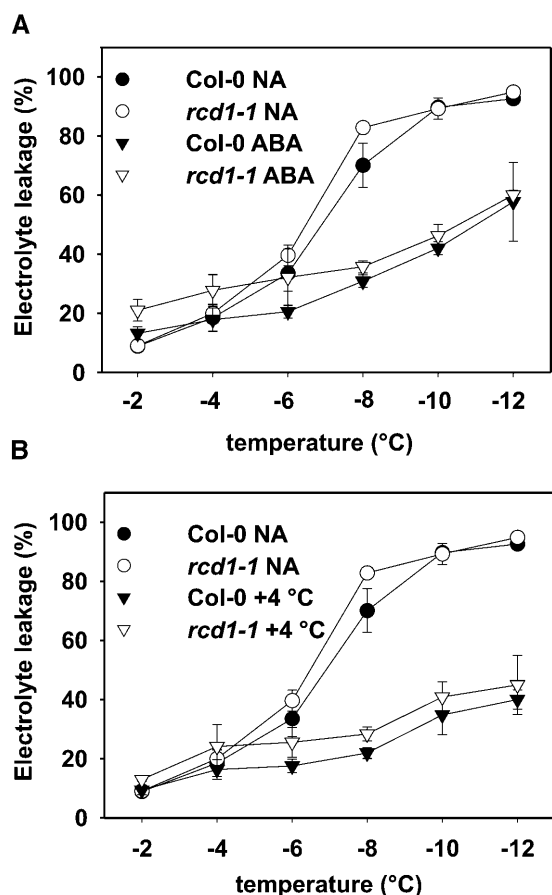


Figure 6. *rcd1-1* Has a Normal Cold Acclimation.

Cold-induced tissue damage in Col-0 and *rcd1-1* after 2 d of treatment with 60 μ M ABA (A) or low temperature (4°C) (B). Damage was measured as ion leakage. The error bars indicate standard deviation of three replicates (four plants each). NA, nonacclimated control.

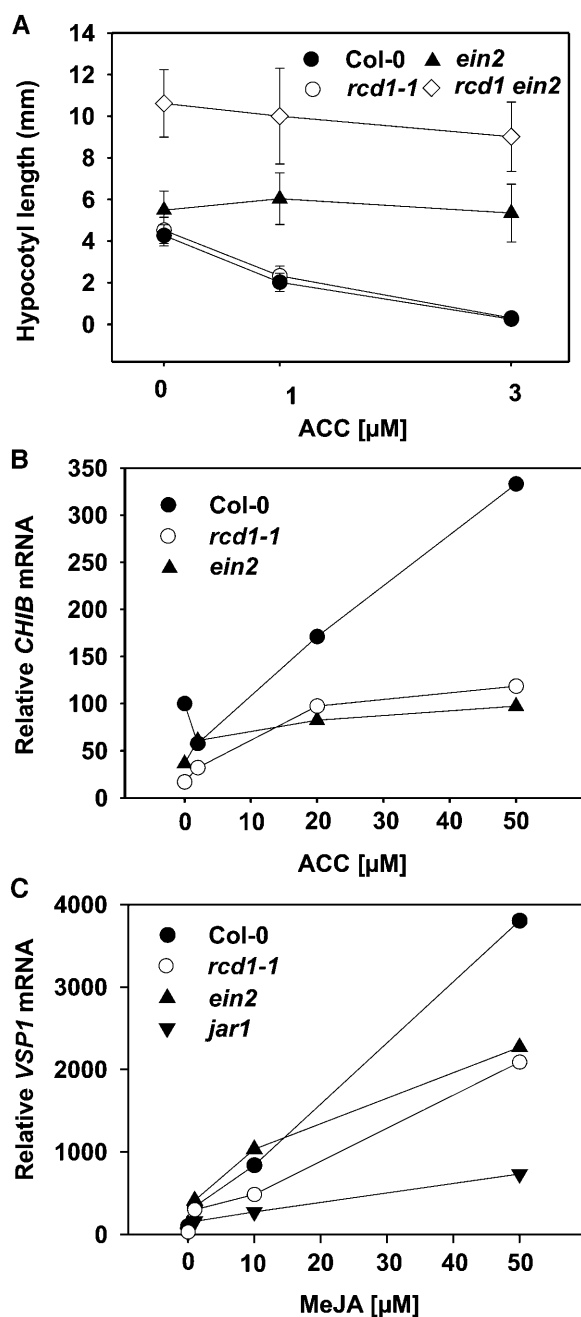


Figure 7. Triple Response Assay and RNA Dot Blot Analysis of ET and Jasmonate Marker Gene Expression.

(A) Triple response assay. Col-0, *rcd1-1*, *ein2*, and *rcd1 ein2* seedlings were grown in darkness for 3 d in the presence of the indicated concentrations of ACC, and the hypocotyl lengths were measured.

(B) *CHIB* expression in 14-d-old in vitro grown Col-0, *rcd1-1*, and *ein2* treated with the indicated concentration of ACC for 48 h. Controls were treated with an equal amount of water.

(C) *VSP1* expression in 14-d-old in vitro grown Col-0, *rcd1-1*, *jar1*, and *ein2* treated with the indicated concentration of MeJA for 48 h. Controls were treated with an equal amount of water.

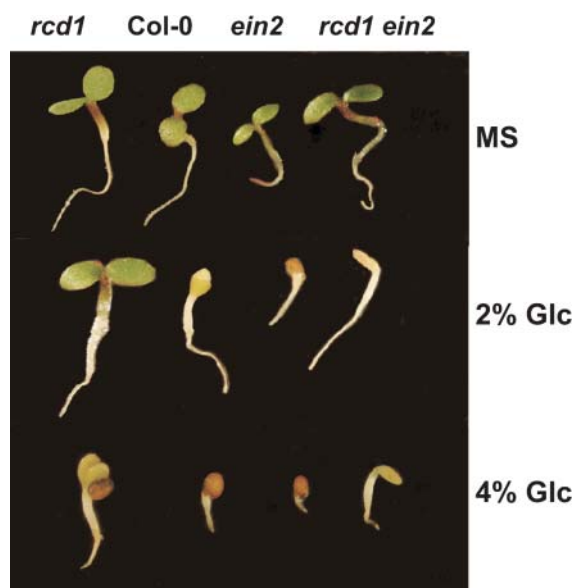


Figure 8. *rcd1-1* Seedling Development Is Partially Insensitive to Glucose.

Three-day-old seedlings of *rcd1-1*, Col-0, ET insensitive, and glucose hypersensitive *ein2* and *rcd1 ein2* double mutant on MS media supplemented with 2 and 4% glucose.

sensitivity, or the lesion propagation in *rcd1*, is more a hormone balance-related runaway process; thus, it might be more accurate to classify *rcd1* as a hormonal mutant instead of an O_3 -sensitive mutant. However, some of the phenotypic characters of *rcd1*, such as the leaf and rosette morphology and faster life cycle, are still visible in double mutants where *rcd1* has been crossed with ET-, SA-, and JA-insensitive mutants (*etr1*, *ein2*, *npr1*, NahG, and *jar1*; K. Overmyer, M. Brosché, and J. Kangasjärvi, unpublished data). Thus, the question as to whether all the developmental phenotypes conferred by *rcd1* are related to hormonal interactions is still an open question and requires further studies.

Interactions among ABA, ET, and ROS

In the triple response assay, *rcd1-1* did not display ET insensitivity compared with *ein2* (Figure 7A). By contrast, ET and JA gene expression responses in *rcd1-1* and *ein2* plants were similarly compromised in comparison to Col-0. Thus, RCD1 seems to affect only a subset of ET responses. The upregulation of ACC oxidase in *rcd1-1* correlates with the higher basal level of ET and the fact that *rcd1-1* overproduces ET in response to O_3 (Overmyer et al., 2000). Based on this misregulation of ET biosynthesis, RCD1 has been proposed to be a regulator of ET production (Wang et al., 2002). ET and jasmonates have mutually antagonistic interactions (Tuominen et al., 2004); thus, the altered JA sensitivity in *rcd1* might be secondary to the increased ET synthesis, which might downregulate JA responses.

Both microarray and macroarray data showed downregulation of cold- and ABA-regulated genes and upregulation of ET-

regulated genes in *rcd1-1*. A further indication for the involvement of RCD1 in relation to both ABA and ET is through the response of *rcd1-1* to glucose (Figure 8), in which *rcd1-1* resembled ABA insensitive and constitutive ET signaling or ET overproducing mutants (León and Sheen, 2003). As described above, *rcd1-1* overproduces ET and has similar defects in the ET and JA marker genes by ACC and JA as *ein2* but bears the opposite glucose sensitivity phenotype, insensitivity rather than hypersensitivity. Furthermore, partial suppression of *ein2* glucose hypersensitivity in the *rcd1 ein2* double mutant (Figure 8) demonstrates the same epistatic relationship between ABA and ET first observed in the double mutants *aba2 etr1* and *aba2 ein2* that exhibit the glucose-insensitive phenotype of *aba2* (León and Sheen, 2003). Mutation in a recently described *ABI8* causes a similar partial suppression of *ein2* glucose hypersensitivity in the *abi8 ein2* double mutant (Brocard-Gifford et al., 2004). At least in the sugar response, the primary effect of the *rcd1-1* mutation seems to be more likely in ABA than ET responses. This also implies that the control of ET biosynthesis by RCD1 may involve ABA.

RCD1 Has a Function in Responses to Drought

One of the RCD1 partner proteins identified by Belles-Boix et al. (2000), DREB2A, is a transcription factor that binds specifically to DRE/CRT, a *cis*-acting element involved in gene expression in response to drought and low-temperature stress (Yamaguchi-Shinozaki and Shinozaki, 1994; Stockinger et al., 1997; Liu et al., 1998; Sakuma et al., 2002). Array analysis indicated that one of the target genes for DREB2a, *RD29A/LTI78*, had lower steady state transcript levels in the *rcd1* mutant (Table 3). Recently, Narusaka et al. (2003) reported the interdependency of the DRE and ABRE (ABA-responsive) elements in the promoter of the *RD29A/LTI78* gene, which contains a single copy of ABRE (ACGTGG/TC), which alone is insufficient for ABA responsiveness (Skriver et al., 1991). However, the expression of *RD29A/LTI78* is strongly induced by exogenous ABA, suggesting that the DRE/CRT-core motif could act as a coupling element of ABRE. Because the truncated *rcd1-1* protein lacks the C-terminal domain that interacts (Belles-Boix et al., 2000) with DREB2A, the reduced ABA sensitivity of *rcd1-1* could result from the mutant protein's inability to activate (or derepress) DREB2A, whose association with the DRE/CRT would be necessary for certain ABRE-dependent ABA responses, such as induction of *RD29A/LTI78*.

rcd1-1 Separates ABA Induction of Cold Acclimation-Related Genes from Cold Acclimation

In spite of the reduced sensitivity of *RAB18* expression to ABA, *rcd1-1* mutant was still able to cold acclimate with both ABA and 4°C treatments. Consequently, ABA-induced expression of cold-induced genes and ABA-induced development of freezing tolerance appear to lie in separate branches of the ABA signal transduction network. Several ABA-dependent and independent pathways exist in response to cold, drought, and salt stress (Ishitani et al., 1997; Heino and Palva, 2003), and *rcd1-1* is a further example of this. To give rise to the desired output,

various signaling pathways have to converge at some point in the signaling networks. The calcium binding protein CBL1 (calci-neurin B-like 1) has been identified as one of these integration points in response to cold, drought, and salt stress (Albrecht et al., 2003; Cheong et al., 2003). Plants overexpressing CBL1 are salt and drought tolerant but freezing sensitive, and loss of CBL1 function makes the plants freezing tolerant and drought and salt sensitive (Albrecht et al., 2003). In a similar way, it is probable that RCD1 plays a role in some branches of ABA signaling and in response to dehydration stress, but it is not involved in ABA-induced cold acclimation and freezing tolerance.

Potential Mode of Action of RCD1

In addition to the gene expression analysis presented in Figures 3 and 4, *RCD1* expression was investigated by searching publicly available microarray experiments. In the abiotic stress and ABA experiments (Seki et al., 2002a, 2002b), available in the RIKEN Arabidopsis Genome Encyclopedia (http://rarge.gsc.riken.go.jp/microarray/microarray_data.pl), *RCD1* was induced twofold to fourfold, which is similar to induction by O_3 . The nuclear localization signal sequences in RCD1 suggest nuclear localization for the protein. These, along with the fact that the interacting partners identified for RCD1 (Belles-Boix et al., 2000) are either known or putative transcription factors, supports the role of RCD1 as a regulator of signal transduction. The domain composition of RCD1 suggests that it does not bind DNA but interacts with other proteins through its N-terminal WWE protein-protein interaction domain (Aravind, 2001) and/or its C-terminal end (Belles-Boix et al., 2000).

In an in vitro binding assay, RCD1 did not hybridize with itself (Lin and Heaton, 2001), suggesting that it functions as a monomer. The downregulation of cold- and ABA-regulated genes in the mutant could be a manifestation of the interaction between RCD1 and its partner proteins not taking place correctly. One possible example for this could be activation of RCD1 by protein X binding to the WWE domain, followed by the binding of the complex to its targets through the C terminus. The RCD1 and SRO proteins have upstream of their C-terminal protein interaction domain a PARP signature [the catalytic domain of poly(ADP-ribose)polymerase] that binds NAD^+ and attaches the ADP-ribose-moiety from NAD^+ to the target molecule. This suggests that the function of the RCD1 (and SRO1) protein could be related to ADP ribosylation. Poly(ADP-ribosylation) by PARPs is a reversible posttranslational modification involved in the regulation of a variety of processes (Smith, 2001). However, RCD1 does not have the conserved zinc-finger domains typical of a classical PARP; thus, it most likely has functions distinct from the classical PARP. The PARP domain protein family also contains a distinct, but less-known subfamily of mono(ADP-ribosyl)transferases that modify their targets by the addition of a single ADP-ribose unit (Smith, 2001; Corda and Di Girolamo, 2003). In animals, mono-ADP-ribosylation of intracellular components by intracellular mono-ADP-ribosyltransferases is just becoming an established process involved in the regulation of protein activity, although so far only genes coding for the extracellular mono-ADP-ribosyltransferases have been identified (Corda and Di Girolamo, 2003). It could be that RCD1 is one

of the predicted (Corda and Di Girolamo, 2003) intracellular plant mono-ADP-ribosyltransferases and inactivates/activates its target proteins by ADP-ribosylation. An intriguing connection is that some bacterial toxins, for example the cholera toxin, are ADP-ribose transferases that increase the activation of trimeric G-protein by ADP-ribosylation. Involvement of G-proteins has been implied in both ABA signaling and in the regulation of ROS production in pathogen responses (Assmann, 2002).

RCD1 has been identified in four separate stress-related circumstances (Belles-Boix et al., 2000; Overmyer et al., 2000; Lin and Heaton, 2001; Fujibe et al., 2004) and displays several pleiotropic phenotypes, most of which can be related to hormonal responses. The alterations in hormonal responses in the *rcd1* mutant suggest that RCD1 could be an integrative node in hormonal signaling and be involved in the hormonal regulation of several specific stress-responsive genes with a biochemical mechanism that has not been described in plants previously.

METHODS

Plant Material and Growth Conditions

Arabidopsis thaliana was grown on 1:1 mixture of peat and vermiculite with subirrigation. Seeds were vernalized for 3 to 5 d. Growth conditions were 23°C/19°C (day/night), 70%/90% relative humidity, under a 12-h photoperiod with 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$ irradiance in controlled growth chambers (Weiss Bio1300; Weiss Umwelttechnik, Reiskirchen-Lindenstruth, Germany) or growth rooms under similar conditions. For specific stress or hormone experiments performed on plants grown in vitro on plates, slightly different conditions may have been used and are listed under the respective experiments. Ozone exposures were started at 9:00 AM with 250 nL L^{-1} for 6 h (sensitivity tests) or 7 h (gene expression analysis) as described (Kiiskinen et al., 1997). Seeds of various Arabidopsis ecotypes and mutants were obtained from the ABRC (<http://www.arabidopsis.org/abrc/>) and the Nottingham Arabidopsis Stock Centre (<http://nasc.nott.ac.uk/>).

Triploid Plant Production

For the production of RCD1/RCD1/*rcd1* triploid plants, *rcd1/rcd1* gl1/gl1 plants were the pollen recipient in a cross with a known tetraploid line in the *co1/co1* gl1/gl1 GL1/GL1 Col-1 background (obtained from the ABRC; stock number CS3432). Only plants identified as true crosses based on the presence of trichomes in the F1 were used for analysis.

Positional Cloning of *rcd1-1*

Visual identification of the recessive *rcd1* habitus was used to select 2000 homozygous *rcd1/rcd1* individuals from more than 10,000 F2 progeny of *rcd1* $\times$ Ler cross. The plants were genotyped with simple sequence length polymorphic and cleaved-amplified polymorphic sequence markers. Candidate genes were amplified from *rcd1-1* using *Pfu* polymerase (Promega, Madison, WI) and sequenced with internal primers. BLAST and PSI-BLAST searches (Altschul et al., 1997) of the nonredundant protein database (National Center for Biotechnology Information) were performed to find homologs of RCD1.

Genetic Complementation

RCD1 and *rcd1-1* cDNAs were prepared from leaf total RNA by RT-PCR according to the manufacturer's instructions (One-Step RT-PCR; Qiagen,

Hilden, Germany) using gene-specific primers (5'-TTACAATCCACCTG-CACCTTC-3' and 5'-ATGGAAGCCAAGATCGTCA-3') and Hot Start Taq DNA polymerase (Promega). PCR products were cloned into pGEMT-Easy vector (Promega), confirmed by sequencing, cloned (*NotI*) into pART27 binary vector (Gleave, 1992), and introduced into *Agrobacterium tumefaciens* strain C58C1pGV2260 by electroporation. Plants were transformed using the floral dip method (Clough and Bent, 1998). Kanamycin-resistant T1 plants were confirmed by PCR and DNA gel blot analyses. As a complementation test, surface-sterilized T2 seeds were germinated on 1% agar MS plates containing 1.0 μ M paraquat. To determine O₃ sensitivity, 21- to 28-d-old T2 plants were exposed to O₃ for 4 h with 300 nL L⁻¹. Cell death was measured by ion leakage from rosette leaves as described in Overmyer et al. (2000).

Reverse Transcriptase-Mediated PCR

Total RNA from *rcd1-1* leaves was used for reverse transcription with FirstChoice RLM-RACE kit (Ambion, Austin, TX) according to the manufacturer's instructions. 5'-RACE PCR was in two steps using nested gene-specific primers (5'-TTACAATCCACCTGCACCTCCTCATGGTCT-3' and 5'-ATCTCCTTCGACTTTGGCTGGTTTGA-3') and nested primers from the 5' RACE adaptor provided in the kit. Annealing temperatures for the reactions were 60°C (outer) and 65°C (inner). For sequencing, the PCR products were cloned into pGEMT-Easy vector.

Gene Expression Analyses

RNA was isolated according to Carpenter and Simon (1998) or using the Qiagen RNEasy plant kit (Qiagen). Gene-specific DNA probes were labeled with α -³²P using Ready-To-Go DNA labeling beads kit (Amersham Biosciences, Buckinghamshire, UK). RNA gel blot and dot blot hybridizations and validation of the specificity and sensitivity of the dot blot hybridizations are described in Overmyer et al. (2000) and Tuominen et al. (2004). The microarray containing ~6500 genes was hybridized with probes prepared from 23-d-old Col-0 and *rcd1-1* RNA. Six biological repeats (each 5 to 10 plants) were pooled into pairs of two, each of the three repeats were labeled with cy3 and cy5 and with the dyes swapped for a total of six hybridizations. The image analysis was with GenePixPro 5.0 (Axon Instruments, Union City, CA). Visually bad spots or areas and low intensity spots were excluded. Low intensity spots were determined as spots where <55% of the pixels had intensity above the background +1 SD in either channel. The GenePixPro 5.0 data was imported into GeneSpring 6.0 (Silicon Genetics, Redwood City, CA) and normalized with the Lowess method. The background subtracted median intensities were used for calculations.

Expression of 92 stress- and defense-related genes was characterized in samples collected from 3-week-old plants with macroarrays described in Overmyer et al. (2000) and Tuominen et al. (2004). Gene expression was quantified by hybridization of a ³²P-labeled cDNA probe prepared from each mRNA sample and normalized by division with the mean expression level of two constitutively expressed genes, *ACT2* (At3g18780) and *ACT8* (At1g49240).

Hormone Treatments

For ABA treatment, sterilized seeds were sown on medium containing half-strength MS salts (Sigma-Aldrich, St. Louis, MO), 0.1% Mes, 2% sucrose, and 0.8% Bacto agar, pH 5.7. After vernalization for 3 d at 4°C, the plates were incubated in controlled growth chambers (Sanyo, Sakata, Japan) with 22°C temperature and 70% relative humidity under a 12-h photoperiod (130 μ mol m⁻² s⁻¹). At 4 d, seedlings were transferred to 12-well plates containing 1.5 mL of the same medium. At 14 d, 200 μ L of ABA, ACC, and/or MeJA solutions was added to the plates. Plants were harvested after 48 h.

Triple Response Assay and Sensitivity to Glucose

For triple response assay, surface-sterilized seeds were sown on MS with 0, 1, or 3 μ M ACC, vernalized for 5 d at 4°C, and incubated in a growth chamber with 22°C temperature and 70% relative humidity in darkness for 3 d. To assess glucose sensitivity, seeds were sown on MS plates supplemented with 0, 2, 4, or 6% glucose and incubated in the same conditions except for constant light for 4 d.

Stomatal Conductance and Water Loss

Leaf stomatal conductance to water vapor was measured with an AP4 steady state diffusion porometer (Delta-T, Cambridge, UK). Three healthy leaves from five different plants were analyzed for each genotype and time point in two replicate measurements. Transpiration from detached leaves was measured as described by Leung et al. (1997).

Freezing Tolerance

Plants grown on agar plates were acclimated by applying ABA to the growth media to a final concentration of 60 μ M or keeping the plants at 4°C in a controlled growth chamber (Sanyo). To test freezing tolerance, at least four rosettes were cut above the root, and their freezing tolerance was tested as described in Tamminen et al. (2001).

Accession Numbers for Data Deposited in a Public Repository

Seeds of the *rcd1-1* mutant have been deposited to the Nottingham Arabidopsis Stock Centre with the stock number N9354. For the cDNA microarray analysis, the complete protocols, array design, experiment design, and raw data files are available from the ArrayExpress Database (<http://www.ebi.ac.uk/arrayexpress/>) with the accession number E-MEXP-77. The nucleotide sequence of the *RCD1* gene containing the *rcd1-1* mutation has been deposited in GenBank with the accession number AY578788 and two cDNA sequences of the splice variants of *rcd1-1* with accession numbers AY578789 and AY578790.

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