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Overexpression of the *RADICAL-INDUCED CELL DEATH1* (*RCD1*) Gene of Arabidopsis Causes Weak *rcd1* Phenotype with Compromised Oxidative-Stress Responses

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***rcd1* is a mutant of *Arabidopsis thaliana* that is more resistant to methyl viologen, but more sensitive to ozone than the wild type. *rcd1-2* is caused by a single nucleotide substitution that results in a premature stop codon at Trp-332. The *rcd1-2* mRNA level does not change significantly with the mutation. Since over-expression of *rcd1-1* cDNA has been shown to bring about an *rcd1*-like phenotype, we created and examined the overexpression lines of *RCD1* by the use of the cauliflower mosaic virus *35S* promoter. The transgenic lines exhibited a weak *rcd1*-like phenotype, although no resistance to methyl viologen was observed. Further, they fully complemented the aberrant *rcd1-2* phenotype. Subcellular localization of RCD1 was examined by transiently expressing green fluorescent protein (GFP) fused with RCD1 in onion epidermal cells. GFP signals are observed as aggregated foci in the inner nuclear matrix-like region.**

Key words: *Arabidopsis thaliana*; methyl viologen (paraquat); ozone; RCD1; subnuclear localization

We isolated an Arabidopsis mutant that can grow in the presence of reactive oxygen species (ROS)-producing methyl viologen (paraquat) in growth medium. It is completely recessive with respect to methyl-viologen resistance.[1)] Genetic mapping showed that our mutation was located near the previously characterized *RADICAL-INDUCED CELL DEATH1* (*RCD1*) locus, of which a mutant, *rcd1-1*, was hypersensitive to another ROS, ozone.[2,3)] Genetic complementation tests indicated that our mutation was a new allele of *RCD1*, *rcd1-2*.[1)] That is, *rcd1* is resistant to ROS produced in chloroplast by promoting gene expression for ROS-scavenging enzymes in chloroplast such as superoxide dismutase and ascorbate peroxidase,[1)] while it is more sensitive to an apoplastic ROS, ozone, than the wild type. Interestingly, the hypersensitivity of *rcd1* to ozone is semi-dominant.[2)]

Molecular genetic study of *RCD1* has revealed that it encodes a plant-specific CEO1 protein[4)] the cDNA of which has been shown to complement an oxidative stress-sensitive yeast strain.[5)] Although the function of RCD1/CEO1 is unknown, it contains a protein–protein interaction domain, the WWE domain, and the catalytic domain of poly(ADP-ribose)polymerase (PARP) (PARP signature).[4,6)] RCD1 has also been shown by yeast two-hybrid assay to bind to ethylene-responsive element-binding protein (EREBP)-like protein in its carboxyl (C)-terminal region.[5)]

In this study, we examine phenotype of transgenic plants overexpressing the *RCD1* gene (*RCD1* OX) to understand better the molecular basis of genetic characteristics of *rcd1* mutation. We also determine the subcellular localization of RCD1 by the use of green fluorescent protein (GFP)-fusion protein. RCD1 is known to contain three putative nuclear localization signals.[5)]

Materials and Methods

Plant materials. Seeds of *Arabidopsis thaliana* ecotype Columbia were surface sterilized, chilled at 4 °C for 2 to 3 d, and sown on 1% (w/v) agar plates containing one-half-strength Murashige and Skoog medium[7)] supplemented with 1% (w/v) sucrose. They were grown at 23 °C under continuous white fluorescent light. When plants were grown in soil or on rock wool, they were watered with 1,000-fold diluted Hyponex fertilizer (Hyponex Japan, Osaka).

Ozone treatment. Ozone fumigation was performed as described previously.[8)] Three-week-old seedlings were

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Abbreviations: C, carboxyl; DAPI, 4′,6-diamidino-2-phenylindole; GFP, green fluorescent protein; OX, overexpression line; PARP, poly(ADP-ribose)polymerase; ROS, reactive oxygen species; RT-PCR, reverse transcription-polymerase chain reaction

exposed to 0.2 ppm ozone for 8 h in a growth chamber at 25 °C and 70% relative humidity under light from metal halide lamps with a photosynthetic photon flux density of 300 μmol m^{-2} s^{-1}. The plants were grown for 16 h under the same conditions without ozone after treatment, then their photographs were taken.

Quantification of chlorophyll. Chlorophylls were extracted from the upper shoots of seedlings with methanol under dark conditions, and their contents were estimated as described previously.[1]

Reverse transcription (RT)-polymerase chain reaction (PCR). Total RNA was prepared from Arabidopsis grown for two weeks in one-half-strength Murashige and Skoog medium[7] with 1% (w/v) sucrose, using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). RT was carried out by Moloney murine leukemia virus reverse transcriptase RNase H^- (ReverTra Ace, Toyobo, Osaka). PCR was conducted with 22 cycles for cDNA obtained from 225 ng of total RNA by Taq polymerase (New England Biolabs, Ipswich, MA, USA) using a pair of primers, 5′-CCAATGCTGAAGGTAATTTGATT-GC-3′ and 5′-CCTTCGACTTTGGCTGGTTTTGAAG-3′ for *RCD1*, and 5′-TGCTTCTAAACTAAAGAGA-CATCG-3′ and 5′-GCTACAAACAAACAAACAAAT-GGA-3′ for the *ACTIN8* gene.[9] PCR products were visualized by staining with ethidium bromide after electrophoresis with 2% agarose.

Genetic mapping. Two cleaved amplified polymorphic sequence (CAPS) markers, F3C3 and F27G20 were generated at coordinates 11571229 and 11629072 of chromosome 1 respectively. For the F3C3 marker, PCR was performed using a pair of primers, 5′-AAGGCAT-TATTAGTCCAAGAGA-3′ and 5′-TAGAAGATGAT-GCAGTTGAGTT-3′. Digestion of the 702-bp-long PCR product with *Sal*I yielded 280- and 422-bp-long fragments in Columbia, whereas the fragment was not digested in Landsberg *erecta*. For the F27G20 marker, PCR was performed with a pair of primers, 5′-TGTC-TGTAACGCAACCAAGGA-3′ and 5′-GATTCATGC-CAAGATTCCCAG-3′. Digestion of the 570-bp-long PCR product with *Bsa*HI resulted in 323- and 247-bp-long fragments in Landsberg *erecta*. By contrast, it was not cleaved in Columbia.

Overexpression lines. A 3.9-kb *RCD1* genomic sequence, which covered entire *RCD1* coding region, was amplified by PCR using a pair of primers, 5′-CACCACCCAATAAGATTTCGACGTT-3′ and 5′-AGGAGAACCACAAAGGCAGGTAG-3′, and cloned in a Gateway entry vector pENTR/D-TOPO (Invitrogen, Carlsbad, CA, USA). The genomic fragment was then inserted into pGWB8 binary vector downstream of the *35S* promoter of cauliflower mosaic virus[10] by the Gateway system (Invitrogen) and introduced into *Agrobacterium tumefaciens* strain pGV2260. The nucleotide sequence was confirmed by sequencing. *A. thaliana* ecotype Columbia was transformed by the flower dipping method.[11] Collected T_1 seeds were sown on the above-mentioned agar plates containing 25 μg/ml hygromycin. Three hygromycin-resistant seedlings were selected and further grown in soil. They looked similar to each other in morphology.

Complementation with overexpression line. rcd1-2 was crossed with *RCD1* OX, and the resulting F_1 plants were allowed to self-fertilize to generate an F_2 population. *rcd1-2* harboring *35S::RCD1* was identified by the use of a CAPS marker for *rcd1-2* mutation and hygromycin resistance. For the *rcd1-2* CAPS marker, PCR was conducted with a pair of oligonucleotide primers, 5′-GTGTGAAGATGGATTCGG-3′ and 5′-TC-TAAGCTGTCTCCCTTT-3′. The 1,532-bp-long PCR product was digested into 955- and 577-bp-long fragments with *Mva*I in the wild type, while no cleavage was observed in *rcd1-2*.

Microscopic examination of GFP-fusion protein. The *CopGFP* sequence[12] was copied from pCop-Green-B vector (Evrogen, Moscow, Russia) by PCR with a pair of primers, 5′-ATGGAATTCATGCCCGCCATGAA-GATCGAGTGC-3′ and 5′-TTATCTAGAATCATCC-GGAGGCGAATGCGATCGGGGTCTTGAA-3′. The product was inserted into pART7[13] at the *Eco*RI/*Sma*I site of its multiple cloning site downstream of the *35S* promoter. The coding region of *RCD1* cDNA was amplified with PCR using a RAFL full-length cDNA clone for *RCD1* as a template and a pair of oligonucleotides, 5′-ATGCTCGAGTCCGGAATGGAAGCC-AAGATCGTCAAGG-3′ and 5′-ATGTCTAGATTAG-AATTCCAATCCACCTGCACCTTCTTC-3′. After digestion with *Xho*I and *Eco*RI, the *RCD1* sequence was inserted into the *Xho*I/*Eco*RI site of the modified pART7, just upstream of the *CopGFP* sequence. The fusion of *35S* promoter, *RCD1* coding sequence and *CopGFP* cut out from the modified pART7 by *Not*I was ligated with the *Not*I-digested pART27.[13] The nucleotide sequence was confirmed by sequencing.

The modified pART27 was introduced into onion (*Allium cepa*) epidermal cells by particle bombardment (PDS-1000/He, Bio-Rad, Hercules, CA, USA) using 1.0-μm gold particles at 1,100 psi. After incubation in Murashige and Skoog medium[7] for 24 h, the onion cells were examined with a confocal laser scanning microscope (LSM410, Zeiss, Oberkochen, Germany).

Results

First, we carried out fine mapping of the *rcd1-2* mutation (Columbia background) by measuring its linkage to two cleaved amplified polymorphic sequence markers of chromosome 1 in an F_2 population obtained by crossing *rcd1-2* with ecotype Landsberg *erecta*. Examining 480 chromosomes, we found only two and

one recombinants at the F3C3 and F27G20 markers respectively. The two markers were separated by about 60 kb, in which eight genes from At1g32170 to At1g32240 were identified. They included *RCD1* (At1g32230),[4] and *rcd1-2* did not complement with an allele of *RCD1*, *rcd1-1*,[2] which we have already reported.[1] We determined the nucleotide sequence of *rcd1-2* and found a single G-to-A transition that created a premature stop codon instead of Trp-332.

To gain molecular insight into the function of *RCD1* and its mutation, we generated overexpression lines of *RCD1* (*RCD1* OX) in Arabidopsis. We examined two independent *RCD1* OXs that accumulated the *RCD1* message in higher abundance than the wild type (Fig. 1A). Because both lines showed essentially the same phenotype, experimental results are given for only one of them. *RCD1* OX was smaller than the wild type, but larger than *rcd1-2* (Fig. 1B, C). When viability was estimated as chlorophyll contents 3 weeks after germination, *rcd1-2* was resistant to 0.2 μM methyl viologen, as reported previously.[1,4] *RCD1* OX was found to be as sensitive to methyl viologen as the wild type ($P = 0.19$ by Student's *t*-test; Fig. 2A). When observed 16 h after exposure to ozone for 8 h, *rcd1-2* developed chlorotic lesion in leaves in contrast to almost no injury in the wild type (Fig. 2B), as reported previously for *rcd1-1*.[2] *RCD1* OX also exhibited ozone damage, but to a lesser extent than *rcd1-2* (Fig. 2B).

The *35S::RCD1* was introduced to *rcd1-2* by crossing in order to determine whether the construct was able to complement the *rcd1-2* defects. *rcd1-2* harboring *35S::RCD1* was as large as the wild type (Fig. 1B, C). It showed no resistance to methyl viologen (Fig. 2A), and as much tolerance to ozone as the wild type (Fig. 2B). Thus, *RCD1* overexpression completely complemented the *rcd1-2* phenotype tested.

Finally, we examined the nuclear localization of RCD1 by transiently introducing *RCD1-GFP* fusion driven by the *35S* promoter into onion epidermal cells by particle bombardment, because its amino acid sequence contains three putative nuclear localization signals at amino acids 20–24, 54–58, and 320–323.[5] Microscopic determination revealed predicted nuclear localization of RCD1-GFP (Fig. 3B), while non-fused GFP localized diffusively in both nucleus and cytoplasm (Fig. 3A). At higher magnifications, the RCD1-GFP signal was found in the inner nuclear matrix or the chromatin sparse region, and appeared as aggregated foci or speckles (Fig. 3C).

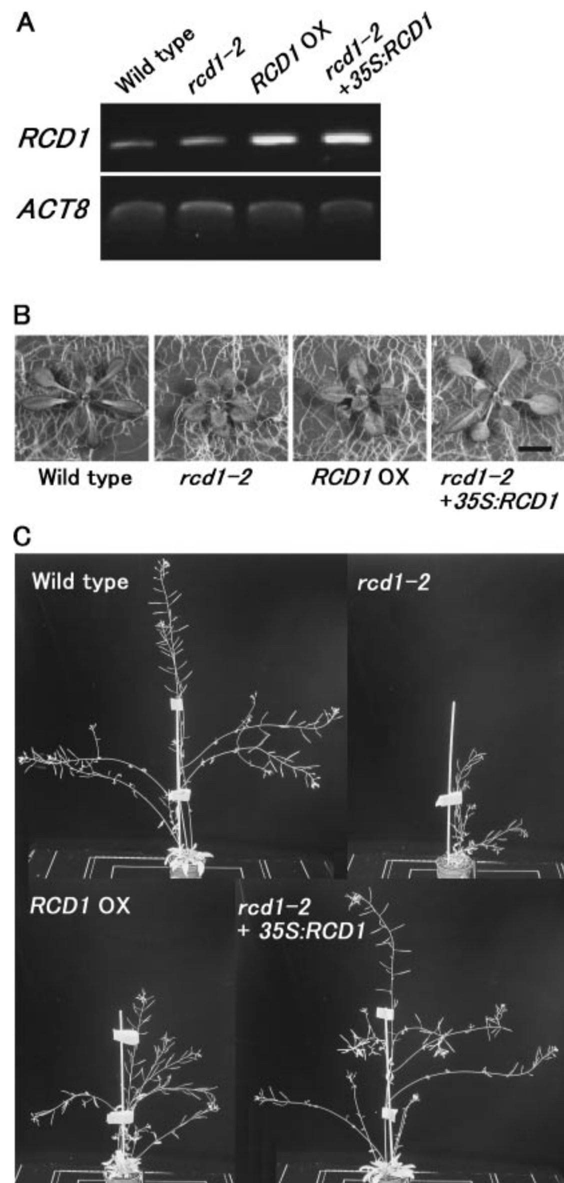

Fig. 1. RT-PCR Analysis of *RCD1* Gene (A), and Morphology of Wild Type, *rcd1-2*, *RCD1* Overexpression Line (*RCD1* OX), and *rcd1-2* Harboring *35S::RCD1* (B, C).

A, RT-PCR analysis of *RCD1* in total RNA prepared from two-week-old plants. PCR products were visualized by staining with ethidium bromide after electrophoresis (upper panel). An actin gene (*ACT8*) was also examined as an internal control (lower panel). B and C, Three-(B) and 7-week-old plants (C) were grown at 23 °C under continuous white light on agar medium and in soil respectively. Bar = 1 cm. Plants in (C) were grown in pots 5.5 cm in diameter.

Discussion

RCD1 protein contains a WWE domain (amino acids 92 to 151) and a PARP signature (amino acids 317 to 416)[4,6] as well as a C-terminal region (amino acids 345 to 589) that can bind to ethylene-responsive element-binding protein-like protein,[5] although their biological significance remains completely unknown in RCD1 function. Because the *rcd1-2* mutation introduces a premature stop codon at amino acid 332, the predicted rcd1-2 protein lacks the C-terminal region as well as most of the PARP domain. Thus *rcd1-2* might be null if either domain is crucial for RCD1 function. It is noteworthy that Arabidopsis PARP proteins (PARP-1 and 2), which probably function in the maintenance of DNA integrity in the nucleus, are also involved in oxidative-stress responses, including a response to

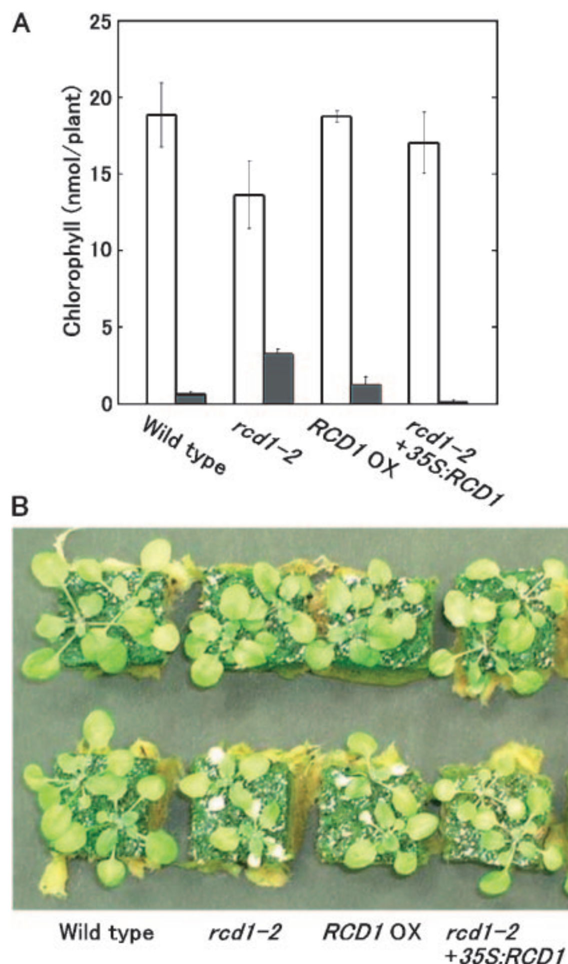

Fig. 2. Sensitivity of *rcd1-2*, *RCD1* OX, and *rcd1-2* Harboring *35S::RCD1* to Methyl Viologen (A) and Ozone (B).

A, Plants were grown for 3 weeks in the absence (open bar) or presence (solid bar) of 0.2 µM methyl viologen, then chlorophyll was extracted from aerial tissues of plants. Values are means ± SE of three independent experiments, in which about 25 seedlings were used. B, Three-week-old plants were grown at 25 °C under continuous white light conditions with (bottom row) or without (top row) 0.2 ppm ozone for 8 h. Photographs were taken 16 h after the ozone treatment.

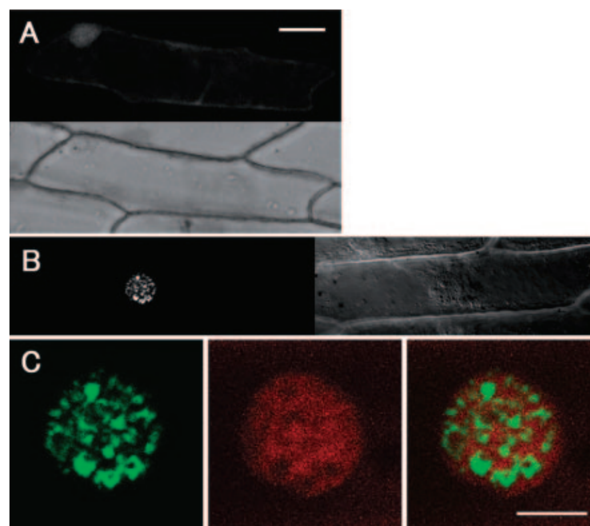

Fig. 3. Subcellular Localization of GFP-Fused RCD1 in Onion Epidermal Cells.

A and B, Fluorescence (top and left) and bright-field (bottom and right) microscope image of non-fused GFP (A) and RCD1-GFP (B). C, Enlarged image of GFP fusion (left) and the nucleus stained with the DNA-binding dye, DAPI (middle), are shown as well as their merged image (right). Bar = 50 and 20 µm in A and C, respectively; A and B are the same in scale.

H_2O_2. mRNA levels of the *PARPs* are induced by exogenously applied H_2O_2 as well as by exposure to ionizing radiation.[14] *RCD1* expression is also promoted by ozone treatment.[4] Although *rcd1-2* is resistant to another abiotic stress, UV-B,[1] *RCD1* expression did not change with irradiation by UV-B for 24 h (data not shown).

rcd1 is completely recessive with respect to methyl-viologen resistance,[1] while it is semidominant with respect to ozone sensitivity.[2] Though *RCD1* OX is as sensitive as the wild type to methyl viologen, ozone sensitivity is compromised in it. This suggests that molecular mechanisms underlying the two ROS-induced phenomena are different. *RCD1* OX is intermediate in size between *rcd1* and the wild type. It is more susceptible to ozone treatment than the wild type, but is less so than *rcd1*. That is, *RCD1* overexpression causes aberrant phenotypes intermediate in strength between *rcd1* and the wild type, but it fully complements *rcd1-2* defects, suggesting that there is an optimum level of RCD1 for proper function, and that either displacement from the optimum can cause the aberrant phenotype.

Overexpression of a mutant (*rcd1-1*) cDNA has been shown to bring about the *rcd1* phenotype to an extent similar to *rcd1-1*.[4] Considering the *rcd1*-like phenotype of triploid *rcd1-1/RCD1/RCD1* plants and the semi-dominant nature of *rcd1-1* with respect to ozone sensitivity, it is concluded that *rcd1-1* is a gain-of-function mutation.[4] *rcd1-1* is a mis-splicing mutation. Sequence analyses of the *rcd1-1* mRNA predict that *rcd1-1* produces either a truncated protein of 397 amino acids containing the amino-terminal 374 amino acids of RCD1, or a 233-amino acid protein that shares C-terminal 214 amino acids of RCD1 (amino acids 376 to 589) due to loss of the entire third exon.[4] The mRNA level of *RCD1* does not differ significantly between the wild type and *rcd1-1*[4] or *rcd1-2* (Fig. 1A). Thus the rcd1-1 or 2 truncated proteins might be able to block normal RCD1 function through a dominant-negative mechanism. Here we showed that *RCD1* overexpression fully complements *rcd1-2* defects. It appears reasonable that an excess amount of RCD1 in *RCD1* OX overcomes such dominant-negative interference. Thus our finding is consistent with the previous conclusion that *rcd1* is a gain-of-function mutation.[4] RCD1 has been predicted to contain a few protein–protein interaction domains, and has been indeed found to bind a transcription factor-like

protein.[5] RCD1 might compete with rcd1 to bind to as-yet-unidentified target proteins in the nucleus.

Recently, subnuclear localization of Arabidopsis nuclear proteins has been investigated systematically, and the RCD1-like distribution has been found for a few of them.[15] At present, each of various functions of nucleus, including transcription, processing, DNA replication and chromatin remodeling, is thought to be organized into discrete subnuclear foci, though study of their function and architecture is just beginning.[16] Observation of RCD1-GFP speckles might indicate significance in nuclear functions. It might be informative that mammalian PARPs have been shown to be localized in the nucleolus, a distinct intranuclear domain.[17] Lastly, it should be noted that the subnuclear localization of RCD1 should be examined by other methods in future studies, because the GFP-fusion gene was overexpressed by the *35S* promoter, which might lead to mislocalization of the product.

In conclusion, we found that an overexpression line of the *RCD1* gene of Arabidopsis displays a phenotype intermediate in severity between *rcd1* and the wild type. It fully complements a point mutation of *RCD1*. We also found the RCD1-GFP fusion protein in small speckles in interchromatin regions of the nucleus, suggesting the involvement of RCD1 in one of numerous nuclear activities.

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