Disease Resistance Gene-Induced Growth Inhibition Is Enhanced by rcd1 Independent of Defense Activation in Arabidopsis*1[C][W][OA]

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Activation of plant immune responses is often associated with an inhibition of plant growth. The molecular mechanisms underlying this fitness cost are unknown. Here, we utilize the autoimmune response mutant suppressor of npr1, constitutive1 (snc1) resulting from an activated form of the Disease Resistance (R) gene to dissect the genetic component mediating growth inhibition in Arabidopsis (Arabidopsis thaliana). The radical-induced cell death1 (rcd1) mutant defective in responses to reactive oxygen species (ROS) was isolated as an enhancer of the snc1 mutant in growth inhibition but not in defense response activation. Similarly, the vitamin C2 (vtc2) and vtc3 mutants defective in ROS detoxification enhanced the growth defects of snc1. Thus, perturbation of ROS status by R gene activation is responsible for the growth inhibition, and this effect is independent of defense response activation. This was further supported by the partial rescue of growth defects of rcd1 snc1 by the respiratory burst oxidase homolog D (rbohD) and rbohF* mutations compromising the generation of ROS burst. Collectively, these findings indicate that perturbation of ROS homeostasis contributes to the fitness cost independent of defense activation.

It is commonly observed that plant growth is inhibited by elevated defense responses. Autoimmune mutants with constitutive defense responses often have reduced growth to different extents compared with the wild type, and these growth defects can be reduced or reverted by inhibition of defense responses (Clarke et al., 2001; Hua et al., 2001; Jambunathan et al., 2001; Shirano et al., 2002; Zhang et al., 2003; Yang and Hua, 2004). Cell expansion and sometimes cell division are reduced while more cell death occurs when defense responses are constitutively activated. Even in the absence of constitutive defense activation, there is a fitness cost associated with harboring disease resistance proteins in the genome (Tian et al., 2003). How defense responses repress plant growth at the molecular level is not well understood. The general assumption is that resources are channeled to defense responses and thus compromise plant growth. In addition, several molecules up-regulated in defense responses have adverse effects on plant growth. For instance, salicylic acid (SA), a signal for systemic acquired resistance (Durrant and Dong, 2004), inhibits plant growth, as revealed by a larger plant with the depletion of SA (Scott et al., 2004). Reactive oxygen species (ROS) generated during defense may also contribute to the growth inhibition. ROS are produced from organelles with a highly oxidizing metabolic activity or with an intense rate of electron flow, including chloroplast, mitochondria, and peroxisomes (Mittler et al., 2004). Conditions that limit CO2 fixation, such as drought, salt, and temperature stress, particularly in combination with high light intensities, enhance ROS production (Mittler, 2002; Foyer and Noctor, 2005; Miller et al., 2008). ROS are also generated in plants during plant-microbe interaction, and the oxidative burst upon pathogen attack is thought to be part of the defense responses (Nanda et al., 2010). It is linked to papilla formation and the assembly of physical barriers in basal resistance. ROS are associated with the hypersensitive response, a form of programmed cell death, to restrict the spread of pathogens upon their recognition by plant Disease Resistance (R) genes. ROS play a dual role in plants as both toxic compounds and important signal transduction molecules (Apel and Hirt, 2004; Mittler et al., 2004; Foyer and Noctor, 2005). Hydroxyl radicals react instantaneously with proteins, lipids, and DNA, causing rapid cell damage. Superoxide (O2−) and hydrogen peroxide (H2O2) can inactivate various

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1 This work was supported by the National Science Foundation (grant no. ISO-0919914 to J.H.), by the National Science Foundation of China (grant no. 31170254 to Y.Z.), and by fellowships from the China Scholarship to B.D. and B.Z.

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www.plantphysiol.org/cgi/doi/10.1104/pp.112.213363
macromolecules directly or be converted to more toxic hydroxyl radicals. ROS can also cross-link Hyp-rich glycoproteins in the cell wall, likely contributing to the resistance of pearl millet (Pennisetum americanum; Deepak et al., 2007). Thus, cell growth inhibition by ROS might result from a combination of toxicity and direct physical limitation on cell expansion. On the other hand, ROS are also the positive regulators of the cell and organ growth under nonstress conditions (Foreman et al., 2003). H2O2 and O2− are thought to be involved in lignin formation in cell walls (Barcelo et al., 2007). The Arabidopsis (Arabidopsis thaliana) RHD2 gene encoding the NADPH oxidase AtroboC is required for ROS accumulation at the tip of the root hair and the elongation of the root hair (Schiefelbein and Somerville, 1990; Grierson et al., 1997). Exogenous application of diphenyleiodonium, an inhibitor of flavoproteins such as NADPH oxidases, inhibits the growth of wild-type root hairs (Dunand et al., 2007; Jones et al., 2007), further supporting a positive role of ROS for root hair development. The distribution of ROS is also found to play a role in the transition from proliferation to differentiation in roots, with O2− accumulating primarily in the meristematic zone whereas H2O2 accumulates in the elongation zone (Tsukagoshi et al., 2010).

The Arabidopsis Radical-Induced Cell Death1 (RCD1) gene is involved in ROS signaling. It has been isolated from various genetic and biochemical screens. The rcd1 mutant is sensitive to ozone and has more expanded cell death compared with the wild type when exposed to ozone (Overmyer et al., 2000). However, it is more tolerant than the wild type to methyl viologen and UV-B that generate ROS in chloroplasts (Fujibe et al., 2004). It is more sensitive to salt but less sensitive to abscisic acid (ABA), ethylene, and methyl jasmonate (Ahlfors et al., 2004). RCD1, therefore, appears to serve as an integration point between hormone signaling and a coordinated ozone response. Under nonstressed growth conditions, the rcd1 mutant plant displays developmental defects, including reduced stature, early flowering, and altered leaf and rosette morphology (Ahlfors et al., 2004). The developmental defects become more severe in the double mutant of RCD1 and its homolog Similar to RCD-One1 (Teotia and Lamb, 2009). RCD1 encodes a protein with a WWE protein-protein interaction domain, three nuclear localization signals, and a poly(ADP-Rib) polymerase domain. RCD1 protein is found by yeast two-hybrid screens to interact with several transcription factors that are involved in both developmental and stress-related processes (Jaspers et al., 2009). It was recently shown to interact with DREB2A to influence senescence and stress responses (Vainonen et al., 2012). These data together indicate that RCD1 is required for ROS signaling and plant growth and development.

Here, we report the isolation of rcd1 as a mutant that enhances the growth defect but not disease resistance of an autoimmune mutant induced by an active form of the R protein. Further genetic characterization indicates that ROS have a major impact on growth defects induced by defense responses and that growth inhibition can be modulated independently from disease resistance.

RESULTS
Isolating an int51 Mutant as an Enhancer of snc1
To understand the molecular mechanisms underlying high-temperature suppression of growth defects in defense response mutants, we screened for temperature-insensitive mutants in the background of the temperature-sensitive autoimmune mutant suppressor of npr1, constitutive1 (snc1). The snc1 mutant has a missense mutation in a Nucleotide Binding-Leu Rich Repeat (NB-LRR) R protein resulting in constitutive activation of the SNC1 protein. It is dwarf at 22°C due to constitutive defense responses (Zhang et al., 2003). However, the mutant phenotype of snc1 exhibited at 22°C is rescued at 28°C due to a suppression of defense responses by the elevated temperature (Yang and Hua, 2004; Fig. 1A). Approximately 30,000 individual M2 plants were screened for mutants showing a dwarf phenotype at 28°C. One such mutant, insensitive to temperature51 (int51) snc1, exhibited a smaller stature than the snc1 single mutant (Fig. 1A). The single mutant of int51 was subsequently identified and confirmed (see below), and it exhibited a mild growth defect at both 22°C and 28°C (Fig. 1A). Rosette sizes were measured for the wild type, the single mutants, and the double mutant at 14 d after germination. At this developmental stage, when the phenotypes were not fully manifested, a synergistic interaction was already observed between int51 and snc1 (Fig. 1B). At 28°C, int51 was 57% of the wild type in size and int51 snc1 was approximately 34% of the wild type or snc1 (Fig. 1B). At 22°C, the snc1 and int51 single mutants were about 60% of the wild type in size and the double mutant was 25% of the wild type (Fig. 1B). The double mutant also showed root growth defects. At 14 d after germination, neither snc1-1 nor int51 single mutants showed drastic reduction of root growth. The double mutant had a much shorter root than either of the single mutants at 28°C but not at 22°C (Fig. 1C). These analyses indicate that int51 has a synergistic interaction with snc1 to affect both leaf and root growth.

The int51 Mutant Is Defective in RCD1
The int51 phenotype is caused by a single nuclear recessive mutation. When int51 snc1 was backcrossed to snc1, the F1 plants exhibited wild-type morphology at 28°C and the F2 progeny segregated both dwarf and wild-type-looking plants at a ratio of approximately 1:3. To identify the int51 mutation, int51 snc1 in Columbia-0 (Col-0) was crossed to the wild-type Landsberg erecta (Ler), and their F2 progeny were used for map-based cloning. By genotyping 1,058 dwarf plants segregated at 28°C, the int51 mutation was positioned to a 28-kb region between markers Ch1.1161 and Ch1.1163 located at 11.614 and 11.642 Mb of chromosome 1, respectively (Fig. 2A; Supplemental Fig. S1A). Sequencing this region in int51 snc1 revealed a single G-to-A transition in the
gene RCD1, resulting in a premature stop codon at Trp-431, and we named this mutant rcd1-5 (Fig. 2B). This allele, like the earlier allele rcd1-1 (Overmyer et al., 2000), showed a slightly dwarf phenotype, with 40% reduction in diameter at both temperatures (Fig. 1, A and B). The rcd1-5 defect could be rescued by transforming an RCD1 complementary DNA under the control of the 35S promoter (Supplemental Fig. S1B), indicating that the growth defect is indeed caused by the rcd1 mutation. RNA-blot analysis revealed a slightly higher expression of the RCD1 transcripts in rcd1-5 than in the wild type (Supplemental Fig. S1C), suggesting a possible feedback regulation of RCD1 itself.

To confirm that the rcd1-5 mutation indeed confers a dwarf phenotype to snc1 at 28°C, we generated a double mutant between rcd1-1 and snc1. The rcd1-1 snc1 double mutant showed the same phenotype to that of int51 snc1: dwarf at 28°C and 22°C, with a more severe phenotype at 22°C (Fig. 2C). Therefore, mutation in RCD1 indeed confers the int51 phenotype. For simplicity, we use rcd1 to refer to rcd1-5 (int51) and rcd1 snc1 to refer to int51 snc1 in the rest of this paper.

The Growth Defect Phenotype of rcd1 snc1 Is Not Due to Enhanced Disease Resistance

We determined whether growth defects of rcd1 snc1 at 28°C are due to enhanced disease resistance, as in a number of int mutants studied earlier (Zhu et al., 2010, 2012; Mang et al., 2012). As the rcd1 single mutant has a slightly dwarfed phenotype, we also asked if this growth defect is associated with enhanced disease resistance. A virulent bacterial pathogen Pseudomonas syringae pv tomato (Pst) DC3000, was used to infect the wild-type, snc1, rcd1, and rcd1 snc1 plants, and its growth in plants was measured at day 0 and day 3 after inoculation (Fig. 3A). At 22°C, wild-type and rcd1 plants supported the same amount of bacterial growth at 3 d after inoculation, while snc1 and rcd1 snc1 showed a similar extent of resistance to pathogen. At 28°C, rcd1 exhibited...
the same susceptibility to the pathogen as the wild type. Disease resistance in sncl was reduced at 28°C than that at 22°C. It had either no increase or a slight increase of resistance to pathogen compared with the wild type. The rcd1 sncl double mutant was not more resistant than the sncl single mutant at 28°C. Thus, rcd1 is as susceptible as the wild type to this bacterial pathogen, and it does not enhance the resistance of sncl at 22°C or 28°C.

Correlated with the disease resistance phenotype, the expression of defense response genes PRI and EDS1 was not affected by the rcd1 mutation at either temperature (Fig. 3B). Similarly, no effect on the level of SA was not affected by the expression of defense response genes while rcd1 sncl accumulated the same amount of SA as the wild type, although rcd1 sncl accumulated the same amount of SA as the sncl single mutant at 22°C. At 28°C, the rcd1 single mutant appeared to accumulate a lower amount of SA, but rcd1 sncl accumulated the same amount of SA as Col-0 and sncl, indicating that rcd1 does not enhance SA accumulation in the wild-type background or the sncl background. The growth defects of rcd1 are not dependent on SA or PAD4, a mediator of defense responses (Wiermer et al., 2005). While nahG and pad4 largely suppressed the sncl phenotype at 22°C, they did not alter the phenotype of rcd1 (Fig. 3D). The rcd1 sncl nahG and rcd1 sncl pad4 plants looked essentially the same as rcd1 plants (Fig. 3D), indicating that PAD4 and SA are required for R activation to inhibit growth. In sum, rcd1 enhances the growth defects of sncl without enhancing its disease resistance, and the growth defects of rcd1 are not due to enhanced disease resistance.

rcd1 Enhances Growth Defects of the Autoimmune Mutant bon1

To investigate whether the rcd1 mutation could enhance the growth defects of other constitutive defense response mutants, we introduced the rcd1 mutation to the autoimmune mutant bonzai1-1 (bon1). The BON1 gene encodes an evolutionarily conserved calcium-binding protein localized to the plasma membrane (Hua et al., 2001; Li et al., 2010) and acts as a negative regulator of SNC1 (Yang and Hua, 2004). Similar to sncl, the bon1 mutant exhibits a temperature-dependent growth and defense phenotype (Yang and Hua, 2004). A double mutant of rcd1 and bon1 was generated, and it exhibited a more severe growth defect than either of the single mutants at 22°C (Supplemental Fig. S2A), indicating an enhancement of the growth defect of bon1 by rcd1. However, the double mutant had a growth phenotype similar to that of rcd1 at 28°C (Supplemental Fig. S2A), suggesting a more complete suppression of the bon1 defects than the sncl defects at high temperatures.

Mutants etc2 and etc3 Enhance the Growth Defects of sncl

RCD1 was proposed to function in ROS signaling, and the enhancement of growth defects of sncl by rcd1 independent of defense activation suggests a synergistic

Figure 3. rcd1 does not enhance disease resistance at 22°C or 28°C in Arabidopsis. A, Growth of Pst DC3000 in Col-0, rcd1, sncl, and rcd1 sncl at 22°C and 28°C. Shown is the growth of bacterial strains at 0 and 3 d post inoculation. Each genotype at each temperature had three biological repeats in each experiment. Values represent means ± SD (n = 3). The letter a indicates a significant difference from the wild-type Col-0 but the same between the two genotypes (P < 0.05 according to Student’s t test). Similar results were obtained in three independent experiments, although sncl and rcd1 sncl, while behaving the same, did not always show significant differences from Col-0 at 28°C at 3 d post inoculation. cfu, Colony-forming units. B, Expression of EDS1 and PRI is not altered by the rcd1 mutation in sncl at either 22°C or 28°C assayed by RNA blots. Ribosomal RNA was used as a loading control. C, Quantification of SA levels in Col-0, sncl, rcd1, and rcd1 sncl plants. Plants were grown on soil for 3 weeks at 22°C and 28°C. Data are means ± SD (n = 3) from three biological repeats. Asterisks indicate significant differences from the wild-type Col-0 (P < 0.05). FWT, Fresh weight.

D, SA and PAD4 are not required for the dwarf phenotype of rcd1. Shown are the wild-type Col-0, rcd1, sncl, rcd1 sncl, sncl NahG, rcd1 NahG, rcd1 sncl NahG, sncl pad4, rcd1 pad4, and rcd1 sncl pad4 plants grown at 22°C and 28°C. [See online article for color version of this figure.]
interaction of the defense response and the ROS response in growth regulation. Therefore, we tested the interaction of \textit{snc1} with several mutants defective in ROS generating or scavenging. L-Ascorbic acid (vitamin C) is an antioxidant that detoxifies ROS, in particular H$_2$O$_2$ (Smirnoff, 2000). Three \textit{vtc} mutants (\textit{vtc1}, \textit{vtc2}, and \textit{vtc3}) were identified as accumulating lower levels of L-ascorbic acid compared with the wild type (Conklin et al., 2000). Double mutants were generated between \textit{snc1} and the three \textit{vtc} mutants, and their growth phenotypes were analyzed at 22°C and 28°C. While neither the \textit{snc1} nor the \textit{vtc} mutants exhibited any growth defects at 28°C, the \textit{vtc2 snc1} and \textit{vtc3 snc1} double mutants, but not the \textit{vtc1 snc1} double mutant, showed more severe growth defects than either single mutant at both temperatures (Fig. 4, A and B; Supplemental Fig. S2).

As the \textit{vtc2} mutant was reported to have elevated cell death and disease resistance (Pavet et al., 2005; Mukherjee et al., 2010), we tested if the growth defect in \textit{vtc2 snc1} at 28°C could be due to an enhanced disease resistance. The growth of the virulent pathogen \textit{Pst} DC3000 was compared with the wild type, \textit{snc1}, \textit{vtc2}, and \textit{vtc2 snc1} (Fig. 4C). Both the \textit{snc1} and \textit{vtc2} mutants had enhanced disease resistance at 22°C but were as susceptible as the wild type at 28°C. However, the \textit{vtc2 snc1} double mutant did not exhibit an enhanced disease resistance compared with the single mutants at either temperature. Thus, similar to \textit{rcl1}, the \textit{vtc2} and probably the \textit{vtc3} mutation enhances the growth defects of \textit{snc1} at 22°C and induces the growth defects of \textit{snc1} at 28°C without enhancing disease resistance.

High Accumulation of H$_2$O$_2$ in \textit{rcl1 snc1}

The dissociation of the effects of defense and growth on \textit{snc1} by \textit{rcl1} and \textit{vtc2} suggests that perturbed ROS production or signaling might be the cause of growth defects in \textit{rcl1 snc1}. To this end, we analyzed the levels of two ROS, H$_2$O$_2$ and O$_2^-$, in the wild type, \textit{snc1}, \textit{rcl1}, and \textit{rcl1 snc1} at 22°C and 28°C. As indicated by staining with 3,3’-diaminobenzidine (DAB), \textit{snc1} but not \textit{rcl1} accumulated a higher level of H$_2$O$_2$ than the wild type at 22°C, and \textit{rcl1 snc1} accumulated an even higher level of H$_2$O$_2$ (Fig. 5A).

At 28°C, no obvious accumulation of H$_2$O$_2$ was observed in \textit{snc1}, \textit{rcl1}, or the wild type, but the \textit{rcl1 snc1} double mutant had a dramatic increase of H$_2$O$_2$ (Fig. 5A). As indicated by staining with nitroblue tetrazolium (NBT), the \textit{rcl1} single mutant had a higher accumulation of O$_2^-$ than the wild type or \textit{snc1}, while the \textit{rcl1 snc1} double mutant had a lower level of O$_2^-$ than in \textit{rcl1} at both temperatures (Fig. 5B). Thus, the enhanced growth defects phenotype in \textit{rcl1 snc1} is correlated with a higher H$_2$O$_2$ level.

Partial Suppression of the Growth Defect of \textit{rcl1 snc1} by \textit{rboh} Mutations

To determine if the high accumulation of H$_2$O$_2$ is the cause of growth defects, we introduced into \textit{rcl1 snc1} mutations that reduce the level of ROS. ROS are generated through metabolic processes as well as by \textit{RBOH} (for respiration burst oxidase homolog) NADPH oxidases (Torres et al., 2005). Among the 10 \textit{RBOH} members, \textit{AtRbohD} and \textit{AtRbohF} are the two expressed throughout most of the plant tissues, and they contribute to innate immune defense and ABA-dependent stomatal opening (Torres et al., 2002; Kwak et al., 2003). Loss-of-function mutants of \textit{rbohD} and \textit{rbohF} were introduced into \textit{rcl1 snc1}. The \textit{rbohF} mutation partially rescued the growth defect of \textit{rcl1 snc1} at 28°C but not 22°C (Fig. 6A). The level of H$_2$O$_2$ was greatly reduced in \textit{rcl1 snc1 rbohF} compared with \textit{rcl1 snc1}, correlating with the growth phenotype (Fig. 6B). The \textit{rbohD} mutation suppressed the \textit{rcl1 snc1} defects at both 22°C and 28°C (Fig. 6C), and this was correlated with a reduction of H$_2$O$_2$ in the triple mutant. These data suggest that \textit{RbohD} and \textit{RbohF} are required for growth inhibition in the \textit{rcl1 snc1} double mutant and that this is likely through the production of H$_2$O$_2$.

DISCUSSION

Plant growth is often inhibited by elevated defense responses, which is readily evident from the dwarf phenotypes exhibited by autoimmune mutants. Blocking defense responses by disrupting signaling components

![Figure 4.](image-url)
such as PAD4, EDS1, and SA often suppresses growth inhibition, indicating that early defense signaling molecules are responsible for both defense and growth. Whether and where the regulation of defense and growth become separated are not known. Here, we isolated an enhancer of autoimmune mutants only in growth inhibition but not in defense activation, revealing a bifurcation in the regulation of two processes after the core defense signaling involving PAD4 and SA (Fig. 7).

Our study indicates that growth inhibition by defense activation is tightly linked to ROS homeostasis and responses and that this effect can be independent of defense responses (Fig. 7). It is striking that snc1 has no activation of defense responses at high temperature and yet snc1 still has a synergistic interaction with rcd1 on growth inhibition at high temperature. This indicates that the activated R gene snc1 has two effects: one is disease resistance induction, which can be suppressed by temperature, and the other is growth inhibition, which can be enhanced by other mutations independently of defense responses. The snc1 growth defect enhancer mutant rcd1 has altered ROS responses and stress responses, suggesting that the second effect of an autoimmune mutant is a change of redox status. This is supported by the enhancement of the growth defect of snc1 by vtc2 and vtc3 mutations, both of which cause lower levels of ROS-detoxifying ascorbic acids.

The level of H$_2$O$_2$ is correlated with the extent of growth inhibition: it is accumulated more in snc1 than in the wild type and even more in rcd1 snc1. The rcd1 snc1 phenotypes were suppressed by mutations in the ROS-generating genes RbohD and RbohF. These NADPH oxidases are responsible for oxidative bursts during pathogen invasion, and they often contribute to cell death but not necessarily disease resistance (Marino et al., 2012). The level of H$_2$O$_2$ was indeed reduced in rboh rcd1 snc1 compared with rcd1 snc1 when growth defects were inhibited, indicating that the high accumulation of H$_2$O$_2$ is at least partially responsible for the growth inhibition. We hypothesize that RBOH oxidases generate ROS in snc1, which leads to growth inhibition when the redox system is further perturbed. Mutation in RbohD but not RbohF suppressed the growth defects of snc1 rcd1 at 22°C, indicating that RbohD has a larger role than RbohF in mediating SNC1 function. This is consistent with earlier observations that the RbohD gene has a larger role than RbohF in most of the defense responses analyzed. We also observed a decrease of O$_2^-$ in the rcd1 snc1 mutant compared with the single mutants. O$_2^-$ has been implicated in promoting growth in roots, as it accumulates at the site of ectopic bulges in the supercentipede1 mutant defective in the RhoGTPase GDP dissociation inhibitor AtRhoGDI1 (Carol et al., 2005). It has yet to be determined whether low O$_2^-$ in rcd1 snc1 contributes to reduced growth and whether it results in high H$_2$O$_2$ through conversion.

In general, there is a synergistic interaction between defense response mutants and ROS mutants. More severe dwarf phenotypes were observed for rcd1 snc1, rcd1 bon1, vtc2 snc1, and vtc3 snc1 at 22°C. However, no enhancement was observed for rcd1 bon1 at 28°C. It is possible that high temperature has a more complete rescue of the bon1 defects than the snc1 defects and, therefore, that rcd1 bon1 behaves like rcd1. Although
vtc1, vtc2, and vtc3 all accumulate less ascorbic acid and VTC1 and VTC2 both encode enzymes for vitamin biosynthesis, vtc1 does not enhance snc1 while vtc2 and vtc3 do. The VTC1-coded enzyme provides GDP-Man, which is used for cell wall carbohydrate biosynthesis and protein glycosylation as well as for ascorbic acid biosynthesis (Conklin et al., 1999). Therefore, the effect of vtc1 is pleiotropic and the interaction of vtc1 with snc1 might be more complex. It is also not yet determined whether these VTC genes have differential expression in response to temperature or defense activation and whether their mutants might perturb ROS homeostasis differently under different cellular or environmental conditions.

Moderately high temperatures such as 28°C can inhibit resistance mediated by R genes such as SNC1 due to its inhibition of R activities, and specific mutant forms of SNC1 can confer disease resistance at 28°C (Zhu et al., 2010). Therefore, potential moderate heat stress and/or perturbation of physiology induced by a moderate high temperature of 28°C are not responsible for the inhibition of disease resistance. However, the stress or perturbation could be enhanced by the loss of the ROS-regulating gene RCD1. This probably explains

Figure 6. Partial suppression of the rcd1 snc1 phenotypes by rbohF and rbohD mutations. A and B, Growth phenotypes (top) and H2O2 accumulation (bottom) of the wild-type Col-0, snc1, rcd1, rbohF, rcd1 snc1, rcd1 rbohF, and rcd1 snc1 rbohF at 22°C (A) and 28°C (B). C and D, Growth phenotypes (top) and H2O2 accumulation (bottom) of the wild-type Col-0, snc1, rcd1, rbohD, rcd1 snc1, rcd1 rbohD, and rcd1 snc1 rbohD at 22°C (C) and 28°C (D). Accumulation of H2O2 in the above plants is indicated by DAB staining.

Figure 7. Model for growth inhibition induced by R genes.
why the \( rcd1 \) \( snc1 \) double mutant had a great reduction in root elongation at 28°C but not at 22°C compared with the wild type and the single mutants (Fig. 1C). This study further demonstrates a general tradeoff between growth and disease resistance. While biotic resistances are often associated with compromises in growth, growth defects, such as in a dwarfing mutant defective in growth hormone signaling, can be associated with resistance to some pathogens (Saville et al., 2012). Furthermore, ABA-deficient mutants have enhanced disease resistance at high temperature (Mang et al., 2012; Zhang et al., 2012). Plants thus have complex systems to tune in to their environment and modulate their growth and responses to ensure best survival and reproduction.

**MATERIALS AND METHODS**

**Plant Material and Growth Conditions**

Arabidopsis (Arabidopsis thaliana) plants were grown on soil at 22°C or 28°C under constant light (approximately 100 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)) with a relative humidity between 40% and 60% for morphological phenotypic and gene expression analysis. Plants for pathogen tests were grown under a 12/12-h photoperiod. Arabidopsis seedlings used for protoplast transformation were grown on solid medium with one-half-strength Murashige and Skoog salts, 2% (w/v) Suc, and 0.8% (w/v) agar under a photoperiod of 8 h of light/16 h of dark.

**Mutant Screen and Map-Based Cloning**

The procedure was similar to one described previously (Mang et al., 2012). The snc1-1 seeds were treated with 0.25% ethane methylsulfonate for 12 h. Approximately 30,000 M2 plants (derived from 3,000 M1 plants) were screened at 28°C for int mutants with the 22°C snc1-1-like dwarf phenotype. The mapping populations for int51 were created by crossing the mutant in the Col-0 accession to the wild type of the Ler accession. Bulked segregation analysis was performed on pools of 40 plants with simple sequence length polymorphism, cleaved-amplified polymorphic sequence, and derived cleaved-amplified polymorphic sequence markers between Col-0 and Ler.

**Transgenic Plant Generation**

Agrobacterium tumefaciens strain GV3101 (Koncz and Schell, 1986) carrying the 35S:RCDI construct was used to transform \( rcd1-5 \) plants via standard floral dipping. Primary transformants were selected on solid medium containing hygromycin.

**RNA Analysis**

The procedure was similar to one described previously (Zhu et al., 2010). Total RNAs were extracted using TRI Reagent (Molecular Research) from leaves of 3-week-old plants. Twenty micrograms of total RNAs per sample was used for RNA gel-blot analysis according to a standard procedure.

**Pathogen Resistance Assay**

Pseudomonas syringae pv. tomato DC3000 was grown 2 to 3 d on King’s B medium and resuspended at 10\(^{8}\) colony-forming units ml\(^{-1}\) in a solution of 10 mm MgCl\(_2\) and 0.02% Silwet L-77. Two-week-old seedlings were dip inoculated with bacteria and kept covered for 1 h before being slowly uncovered. The amount of bacteria in plants was analyzed at 1 h after dipping (day 0) and 3 d after dipping (day 3). Three seedlings were pooled, and three pools were measured for bacteria growth in each genotype and condition.

**DAB and NBT Staining**

The Col-0, snc1, rcd1, and \( rcd1 \) \( snc1 \) plants were grown on soil at 22°C or 28°C for 2 or 3 weeks. Production of \( \text{H}_2\text{O}_2 \) was assayed by DAB staining. DAB was dissolved in 50 mls Tris-acetate (pH 5.0) at a concentration of 1 mg mL\(^{-1}\). Whole seedlings were placed in the DAB solution and vacuum infiltrated until the tissues were soaked. The seedlings were then incubated at room temperature in the dark for 24 h before the tissues were cleared by several rounds of ethanol (75%). Production of O\(_{2}^-\) was detected by soaking the whole plant in 50 mls Tris-acetate (pH 5.0) at a concentration of 500 mls NBT for 30 min.

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure S1** Characterization of the \( rcd1-5 \) allele.

**Supplemental Figure S2** Growth phenotypes of \( rcd1 \) \( bon1 \) and \( rcd1 \) \( snc1 \).

**ACKNOWLEDGMENTS**

We thank the Arabidopsis Biological Resource Center for \( rcd1-1 \), \( rbolD \), and \( rbolH \) mutant seeds, Tiana Blomster and Kirk Overmyer for the 35S:RCDI construct, and Jed Spark, Kim Spark, Weiqiang Qian, and Zhilong Bao for assistance with mutant characterization.

**LITERATURE CITED**


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