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 CO_2 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 (O_2^-) and hydrogen peroxide (H_2O_2) can inactivate various

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). H_2O_2 and O_2^- are thought to be involved in lignin formation in cell walls (Barceló et al., 2007). The Arabidopsis (*Arabidopsis thaliana*) *RHD2* gene encoding the NADPH oxidase AtrbohC 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 diphenyleneiodonium, 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 O_2^- accumulating primarily in the meristematic zone whereas H_2O_2 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* (L*er*), 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
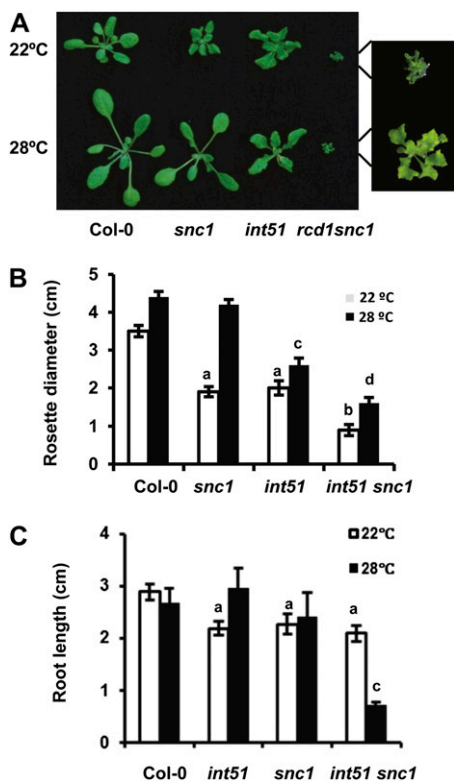
Figure 1. The *int51 snc1* double mutant has reduced growth at both 28°C and 22°C. A, Growth phenotypes of wild-type Col-0, *snc1 int51*, and *int51 snc1* at 22°C and 28°C before bolting. B, Diameter of rosettes of Col-0, *snc1 int51*, and *int51 snc1* at 2 weeks after germination. C, Root length of the above plants grown on vertical plates 2 weeks after germination. A total of 20 and 25 seedlings were measured for A and B, respectively. Values represent means ± SD. Letters (a, b, c) indicate significant differences from the wild-type Col-0, and different letters indicate differences between different mutant genotypes ($P < 0.05$ according to Student's *t* test). [See online article for color version of this figure.]

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
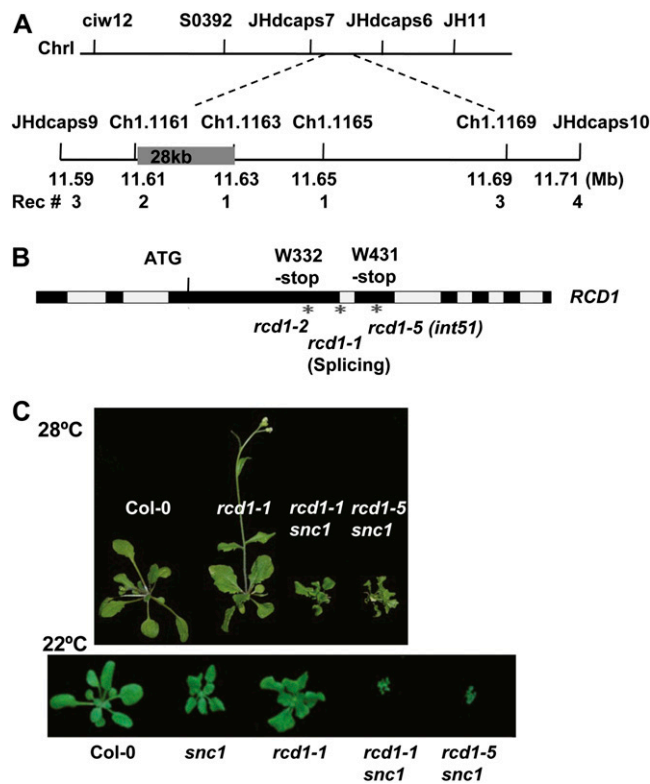

Figure 2. The *int51* mutant is a new allele of *RCD1*. A, Map-based cloning of the *INT51* gene. Shown are the molecular markers used for mapping, their positions on chromosome I (ChrI), and the number of recombinants (Rec #) at each molecular marker. B, A mutation was identified in the *RCD1* gene in the *int51* mutant. This G-to-A substitution leads to the change of Trp-431 to a stop codon. Exons are in black blocks, and introns are in gray blocks. The asterisks indicate different *rcd1* alleles. *int51* is named as *rcd1-5*. C, The *rcd1-1 snc1* double mutant displays a dwarf phenotype similar to *rcd1-5 snc1*. Shown are 3-week-old plants at 28°C. [See online article for color version of this figure.]

the same susceptibility to the pathogen as the wild type. Disease resistance in *snc1* was reduced at 28°C than at 22°C. It had either no increase or a slight increase of resistance to pathogen compared with the wild type. The *rcd1 snc1* double mutant was not more resistant than the *snc1* 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 *snc1* at 22°C or 28°C.

Correlated with the disease resistance phenotype, the expression of defense response genes *PR1* and *EDS1* was not affected by the *rcd1* mutation at either temperature (Fig. 3B). Similarly, no effect on the level of SA was observed for the *rcd1* mutation (Fig. 3C). The *rcd1* mutant accumulated the same amount of SA as the wild type, while *rcd1 snc1* accumulated the same amount of SA as the *snc1* single mutant at 22°C. At 28°C, the *rcd1* single mutant appeared to accumulate a lower amount of SA, but *rcd1 snc1* accumulated the same amount of SA as Col-0 and *snc1*, indicating that *rcd1* does not enhance SA accumulation in the wild-type background or the *snc1* 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 *snc1* phenotype at 22°C, they did not alter the phenotype of *rcd1* (Fig. 3D). The *rcd1 snc1 nahG* and *rcd1 snc1 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 *snc1* 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 *snc1*, 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 *snc1* defects at high temperatures.

Mutants *vtc2* and *vtc3* Enhance the Growth Defects of *snc1*

RCD1 was proposed to function in ROS signaling, and the enhancement of growth defects of *snc1* by *rcd1* independent of defense activation suggests a synergistic
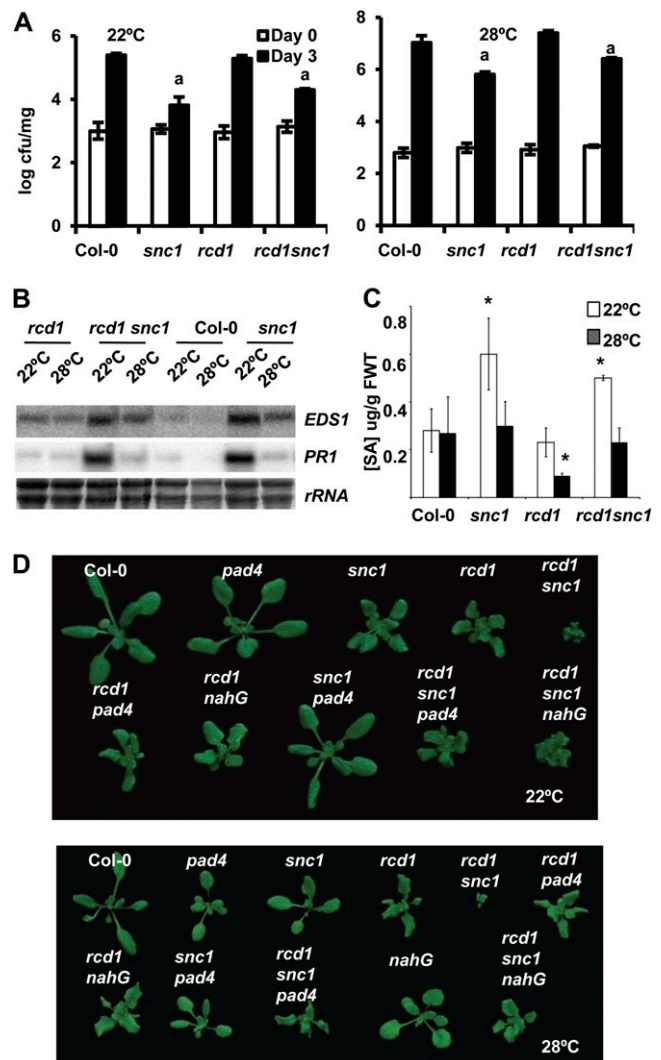

Figure 3. *rcd1* does not enhance disease resistance at 22°C or 28°C in Arabidopsis. A, Growth of *Pst* DC3000 in Col-0, *rcd1*, *snc1*, and *rcd1 snc1* at 22°C and 28°C. Shown is the growth of bacterial strains at 0 and 3 d post inoculation. Each genotype at each temperature had three biological repeats 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 *snc1* and *rcd1 snc1*, 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 *PR1* is not altered by the *rcd1* mutation in *snc1* 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, *snc1*, *rcd1*, and *rcd1 snc1*. 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*, *snc1*, *rcd1 snc1*, *snc1 NahG*, *rcd1 NahG*, *rcd1 snc1 NahG*, *snc1 pad4*, *rcd1 pad4*, and *rcd1 snc1 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 *snc1* with several mutants defective in ROS generating or scavenging. L-Ascorbic acid (vitamin C) is an antioxidant that detoxifies ROS, in particular H_2O_2 (Smirnoff, 2000). Three *vtc* mutants (*vtc1*, *vtc2*, and *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 *snc1* and the three *vtc* mutants, and their growth phenotypes were analyzed at 22°C and 28°C. While neither the *snc1* nor the *vtc* mutants exhibited any growth defects at 28°C, the *vtc2 snc1* and *vtc3 snc1* double mutants, but not the *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 *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 *vtc2 snc1* at 28°C could be due to an enhanced disease resistance. The growth of the virulent pathogen *Pst* DC3000 was compared with the wild type, *snc1*, *vtc2*, and *vtc2 snc1* (Fig. 4C). Both the *snc1* and *vtc2* mutants had enhanced disease resistance at 22°C but were as susceptible as the wild type at 28°C. However, the *vtc2 snc1* double mutant did not exhibit an enhanced disease resistance compared with the single mutants at either temperature. Thus, similar to *rcd1*, the *vtc2* and probably the *vtc3* mutation enhances the growth defects of *snc1* at 22°C and induces the growth defects of *snc1* at 28°C without enhancing disease resistance.

High Accumulation of H_2O_2 in *rcd1 snc1*

The dissociation of the effects of defense and growth on *snc1* by *rcd1* and *vtc2* suggests that perturbed ROS production or signaling might be the cause of growth defects in *rcd1 snc1*. To this end, we analyzed the levels of two ROS, H_2O_2 and O_2^-, in the wild type, *snc1*, *rcd1*, and *rcd1 snc1* at 22°C and 28°C. As indicated by staining with 3,3'-diaminobenzidine (DAB), *snc1* but not *rcd1* accumulated a higher level of H_2O_2 than the wild type at 22°C, and *rcd1 snc1* accumulated an even higher level of H_2O_2 (Fig. 5A). At 28°C, no obvious accumulation of H_2O_2 was observed in *snc1*, *rcd1*, or the wild type, but the *rcd1 snc1* double mutant had a dramatic increase of H_2O_2 (Fig. 5A). As indicated by staining with nitroblue tetrazolium (NBT), the *rcd1* single mutant had a higher accumulation of O_2^- than the wild type or *snc1*, while the *rcd1 snc1* double mutant had a lower level of O_2^- than in *rcd1* at both temperatures (Fig. 5B). Thus, the enhanced growth defects phenotype in *rcd1 snc1* is correlated with a higher H_2O_2 level.

Partial Suppression of the Growth Defect of *rcd1 snc1* by *rboh* Mutations

To determine if the high accumulation of H_2O_2 is the cause of growth defects, we introduced into *rcd1 snc1*
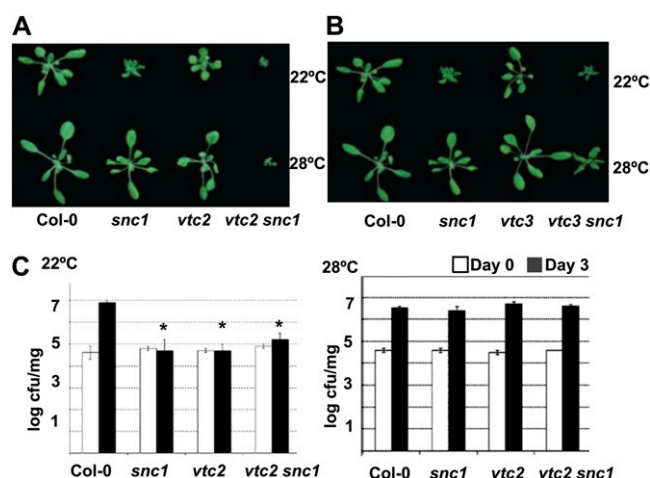

Figure 4. The *vtc* mutations enhance growth defects but not disease resistance of *snc1*. A, The *vtc2* mutation enhances growth defects of *snc1* at both 22°C and 28°C. Shown are Col-0, *snc1*, *vtc2*, and *vtc2 snc1* plants grown on soil for 2 weeks. B, The *vtc3* mutation enhances growth defects of *snc1* at both 22°C and 28°C. Shown are Col-0, *snc1*, *vtc3*, and *vtc3 snc1* plants grown on soil for 2 weeks. C, Disease resistance to *Pst* DC3000 is not enhanced in the *vtc2 snc1* double mutant compared with the *snc1* single mutant at 22°C or 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). Asterisks indicate significant differences from the wild-type Col-0 ($P < 0.05$ according to Student's *t* test). Similar results were obtained in three independent experiments. cfu, Colony-forming units. [See online article for color version of this figure.]

mutations that reduce the level of ROS. ROS are generated through metabolic processes as well as by *RBOH* (for respiration burst oxidase homolog) NADPH oxidases (Torres et al., 2005). Among the 10 *RBOH* members, *AtRbohD* and *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 *rbohD* and *rbohF* were introduced into *rcd1 snc1*. The *rbohF* mutation partially rescued the growth defect of *rcd1 snc1* at 28°C but not 22°C (Fig. 6A). The level of H_2O_2 was greatly reduced in *rcd1 snc1 rbohF* compared with *rcd1 snc1*, correlating with the growth phenotype (Fig. 6B). The *rbohD* mutation suppressed the *rcd1 snc1* defects at both 22°C and 28°C (Fig. 6C), and this was correlated with a reduction of H_2O_2 in the triple mutant. These data suggest that *RbohD* and *RbohF* are required for growth inhibition in the *rcd1 snc1* double mutant and that this is likely through the production of H_2O_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 5. The *rcd1 snc1* mutant accumulates a higher level of H_2O_2. A, H_2O_2 is increased in the *rcd1 snc1* double mutant at both 22°C and 28°C. Shown are DAB staining of the wild-type Col-0, *rcd1*, *snc1*, and *rcd1 snc1* grown at two temperatures. B, O_2^- is decreased in the *rcd1 snc1* double mutant at both 22°C and 28°C. Shown is NBT staining of the wild-type Col-0, *rcd1*, *snc1*, and *rcd1 snc1* grown at two temperatures.
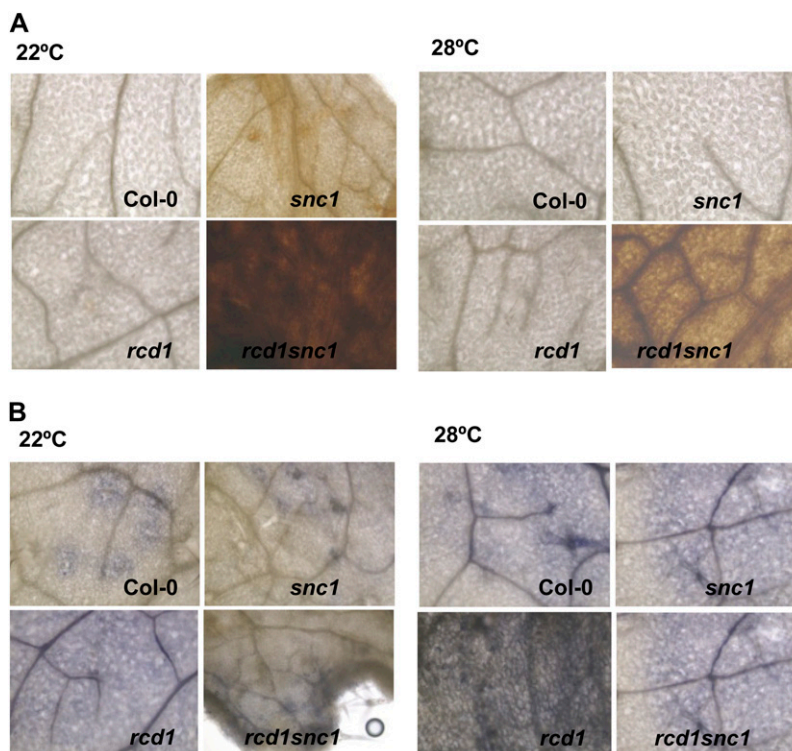
such as PAD4, EDS1, and SA often suppresses growth inhibition, indicating that early defense signaling molecules are responsible for both defense and growth. Whether and where the regulation of defense and growth become separated are not known. Here, we isolated an enhancer of autoimmune mutants only 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_2O_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_2O_2 was indeed reduced in *rboh rcd1 snc1* compared with *rcd1 snc1* when growth defects were inhibited, indicating that the high accumulation of H_2O_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 *suprecentipede1* 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_2O_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
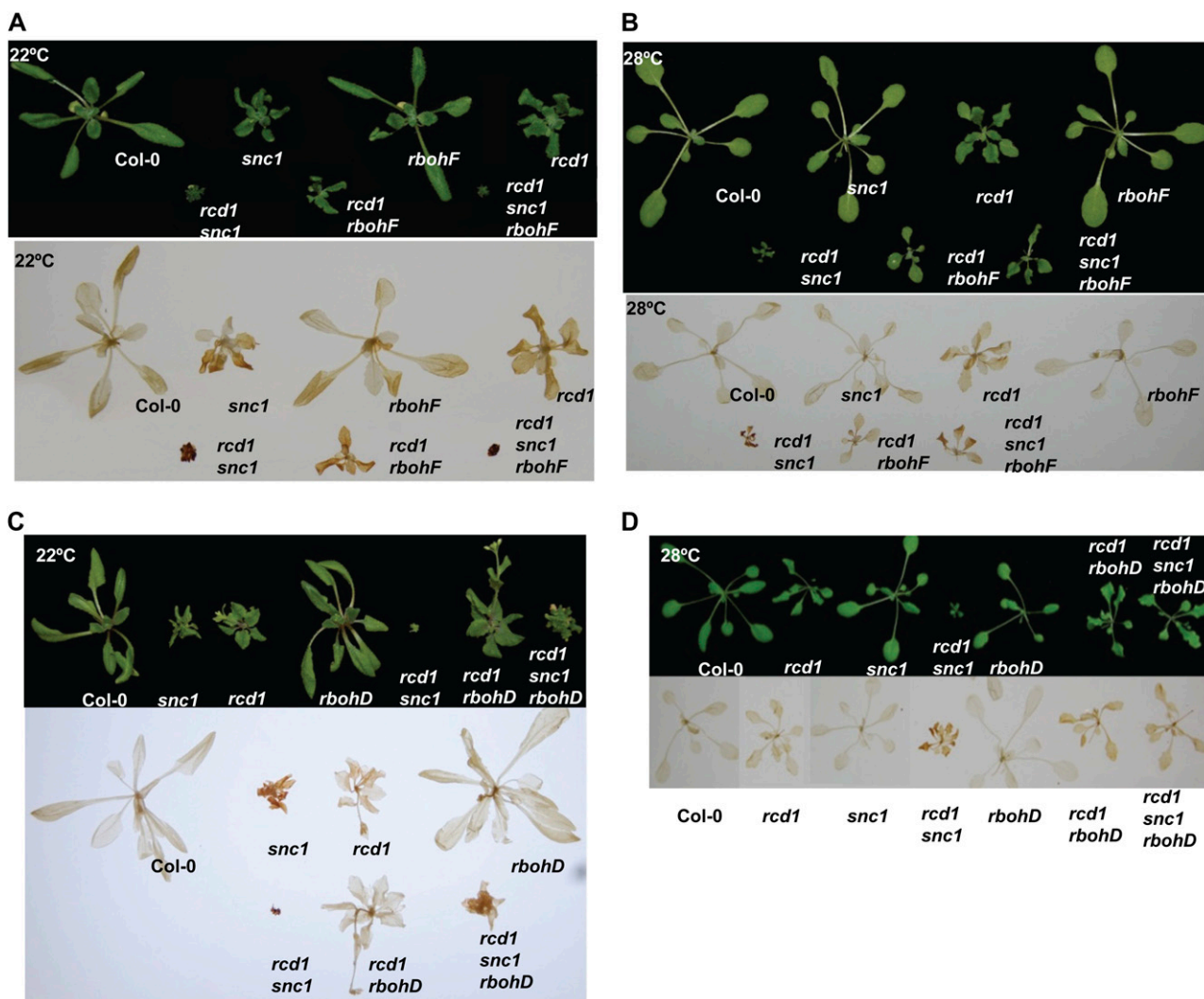
Figure 6. Partial suppression of the *rcd1 snc1* phenotypes by *rbohF* and *rbohD* mutations. A and B, Growth phenotypes (top) and H$_2$O$_2$ accumulation (bottom) of the wild-type Col-0, *snc1*, *rcd1*, *rbohF*, *rcd1 snc1*, *rcd1 rbohF*, and *rcd1 snc1 rbohF* at 22°C (A) and 28°C (B). C and D, Growth phenotypes (top) and H$_2$O$_2$ 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 H$_2$O$_2$ in the above plants is indicated by DAB staining.

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
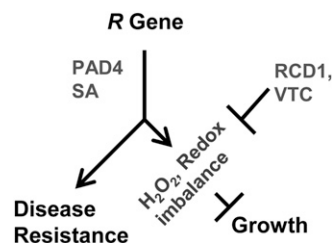

Figure 7. Model for growth inhibition induced by *R* genes.

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 can have enhanced disease resistance at high temperature (Mang et al., 2012; Zhu 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 μmol m^{-2} 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 L*er* 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 L*er*.

Transgenic Plant Generation

Agrobacterium tumefaciens stain GV3101 (Koncz and Schell, 1986) carrying the 35S:RCD1 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 10^5 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 H$_2$O$_2$ was assayed by DAB staining. DAB was dissolved in 50 mM Tris-acetate (pH 5.0) at an 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 mM Tris-acetate (pH 5.0) at a concentration of 500 mM 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 *vtc1 snc1*.

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