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A Cluster of Disease Resistance Genes in *Arabidopsis* Is Coordinately Regulated by Transcriptional Activation and RNA Silencing [W]

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The *RPP5* (for *recognition of Peronospora parasitica 5*) locus in the *Arabidopsis thaliana* Columbia strain contains a cluster of paralogous disease *Resistance* (*R*) genes that play important roles in innate immunity. Among the *R* genes in this locus, *RPP4* confers resistance to two races of the fungal pathogen *Hyaloperonospora parasitica*, while activation of *SNC1* (for *suppressor of npr1-1, constitutive 1*) results in the resistance to another race of *H. parasitica* and to pathovars of the bacterial pathogen *Pseudomonas syringae* through the accumulation of salicylic acid (SA). Here, we demonstrate that other Columbia *RPP5* locus *R* genes can be induced by transgenic overexpression of *SNC1*, which itself is regulated by a positive amplification loop involving SA accumulation. We also show that small RNA species that can target *RPP5* locus *R* genes are produced in wild-type plants and that these *R* genes can be cosuppressed in transgenic plants overexpressing *SNC1*. Steady state expression levels of *SNC1* increase in some mutants (*dcl4-4*, *ago1-36*, and *upf1-5*) defective in RNA silencing as well as in transgenic plants expressing the P1/Helper Component-Protease viral suppressor of RNA silencing. However, steady state levels of small RNA species do not change in mutants that upregulate *SNC1*. These data indicate many Columbia *RPP5* locus *R* genes can be coordinately regulated both positively and negatively and suggest that the *RPP5* locus is poised to respond to pathogens that disturb RNA silencing.

INTRODUCTION

Resistance (*R*) genes are used to recognize specific pathogens with cognate *avirulence* genes and initiate defense signaling that results in disease resistance (Dangl and Jones, 2001; Martin et al., 2003). The proteins encoded by the largest class of *R* genes carry a nucleotide binding site–leucine rich repeat (NBS-LRR) domain, and the N terminus shares similarity with either a coiled-coil domain or a *Drosophila* Toll/mammalian interleukin 1 receptor (TIR). The LRR domain seems to mediate specificity in pathogen recognition, while the N-terminal TIR or coiled-coil motif is likely to play a role in downstream signaling. Some *R* genes show a low steady state expression level in the absence of pathogens but can be induced by a positive feedback mechanism in which salicylic acid (SA) accumulation is required (Shirano et al., 2002; Xiao et al., 2003; Yang and Hua, 2004).

The *Arabidopsis thaliana* Columbia *RPP5* (for *recognition of Peronospora parasitica 5*) locus is comprised of seven TIR-NBS-LRR class *R* genes, which are interspersed with three related sequences and two non-*R* genes (Noel et al., 1999) (see Supplemental Figure 1 online). All of the *R* genes and related sequences in the locus are more closely related to each other than any other *R* gene in the genome, consistent with the hypothesis that the locus was generated by local duplications and rearrangements (Baumgarten et al., 2003; Meyers et al., 2003). *R* genes found in this locus in the Columbia haplotype are highly diverged in nucleotide sequences from those in the Landsberg or Wassilewskija haplotype (Noel et al., 1999; Yang and Hua, 2004). Two *R* genes in the *RPP5* locus, *RPP4* and *SNC1* (for *suppressor of npr1-1, constitutive 1*), have been demonstrated to be functional in disease resistance against bacterial and fungal pathogens (Stokes et al., 2002; van der Biezen et al., 2002; Zhang et al., 2003; Yang and Hua, 2004). The gain-of-function mutation *snc1* is likely to increase SNC1 activity and thereby causes elevated disease resistance and dwarfism (Zhang et al., 2003). Two other mutations mapped to the *RPP5* locus, *cpr1* (for *constitutive expresser of PR genes 1*) and *bal*, display phenotypes similar to *snc1* and exhibit a high degree of phenotypic instability after an exposure to ethyl methanesulfonate (Bowling et al., 1994; Stokes et al., 2002; Stokes and Richards, 2002). Even though the upregulation of *SNC1* was reported in both mutants, neither the molecular nature of the mutations responsible for *SNC1* activation nor the mechanism of phenotypic reversion has been characterized (Stokes et al., 2002; Yang and Hua, 2004).

As a first step to characterize *bal* and *cpr1* mutants, we investigated how *RPP5* locus *R* genes are regulated transcriptionally and posttranscriptionally. RNA silencing at the posttranscriptional level is conserved in diverse eukaryotic organisms and is mediated by small RNA (small interfering RNA [siRNA] and microRNA [miRNA]) that is complementary to target mRNA transcripts (Baulcombe, 2004). RNA silencing processing in plants involves (1) generation of double-stranded RNA (dsRNA), the formation of which may require RNA-dependent

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RNA polymerases (RDRs) (Dalmay et al., 2000; Mourrain et al., 2000; Xie et al., 2001, 2004); (2) production of 21- to 24-nucleotide small RNA by Dicer-like proteins (DCLs) (Park et al., 2002; Xie et al., 2004; Gasciolli et al., 2005; Yoshikawa et al., 2005); and (3) small RNA-guided slicing of target RNA by Argonaute family proteins (AGOs) (Baumberger and Baulcombe, 2005; Qi et al., 2005; Adenot et al., 2006; Qi et al., 2006).

The complexity of the RNA silencing machinery is exemplified by the model plant *Arabidopsis*, which has six RDRs, four DCLs, and 10 AGOs. Specialized but partially overlapping functions were observed among the four DCLs. DCL1 is almost exclusively responsible for the production of miRNA (Park et al., 2002; Rajagopalan et al., 2006). However, loss of one or more functions of DCL2, DCL3, and DCL4 can be partially substituted by other DCLs, although there is a preference or hierarchy in the use of specific DCLs for a given process (Gasciolli et al., 2005; Deleris et al., 2006; Fusaro et al., 2006; Henderson et al., 2006). The few functionally characterized AGOs and RDRs show diversified but still overlapping roles in RNA silencing (Fagard et al., 2000; Mourrain et al., 2000; Xie et al., 2001; Zilberman et al., 2003; Vazquez et al., 2004b; Baumberger and Baulcombe, 2005; Borsani et al., 2005; Katiyar-Agarwal et al., 2006; Qi et al., 2006; Kasschau et al., 2007). A wide range of processes in *Arabidopsis*, such as development, flowering time, stress tolerance, and resistance to bacterial and viral pathogens, are regulated by RNA silencing (Liu et al., 2004; Mallory et al., 2004a, 2004b; Vaucheret et al., 2004; Vazquez et al., 2004b; Borsani et al., 2005; Gasciolli et al., 2005; Deleris et al., 2006; Henderson et al., 2006; Katiyar-Agarwal et al., 2006).

Here, we report that several *RPP5* locus *R* genes, including *RPP4* and *SNC1*, are coordinately regulated by at least two different mechanisms. These *R* genes are positively regulated by transcriptional activation and negatively regulated by RNA silencing. We also found that *SNC1* can be upregulated by mutations abrogating RNA silencing and overexpression of a viral RNA silencing suppressor. Our results suggest that the *RPP5* locus, which is important for innate immunity, can be activated in response to challenges by plant pathogens that disturb RNA silencing.

RESULTS

Transgenic Overexpression of *SNC1* Induces Two Distinct Morphological Syndromes Depending on Transgene Dosage

As reported previously, overexpression of a *SNC1* genomic clone under the control of the strong 35S promoter can induce a *bal*-like morphology (twisted leaves and dwarf stature) (Stokes et al., 2002). Further characterization of transgenic plants revealed that the plants displaying *bal*-like phenotypes were hemizygous for the transgene and produced progeny with either wild-type, *bal*-like, or stunted (very small with unexpanded leaves) phenotypes in the absence of selection for the kanamycin resistance gene marker (Figure 1A). We found that progeny exhibiting wild-type phenotypes lacked the *P35S:SNC1* transgene. By contrast, individuals with *bal*-like phenotypes carried the transgene, and their progeny contained plants with three distinctive phenotypes conforming to a 1:2:1 ratio (22 wild-type:48 *bal*-like:21 stunted plants). Stunted plants in segregating families also carried the *P35S:SNC1* transgene, and they eventually developed a wild-type morphology after 3 weeks. This delayed normal growth syndrome bred true over three generations, suggesting that this syndrome is a consistent, reproducible phenotype of *P35S:SNC1* homozygotes. Moreover, we observed a *bal*-like phenotype among all F1 progeny obtained from crosses between wild-type plants and *P35S:SNC1* transgenic plants exhibiting the delayed normal growth syndrome.

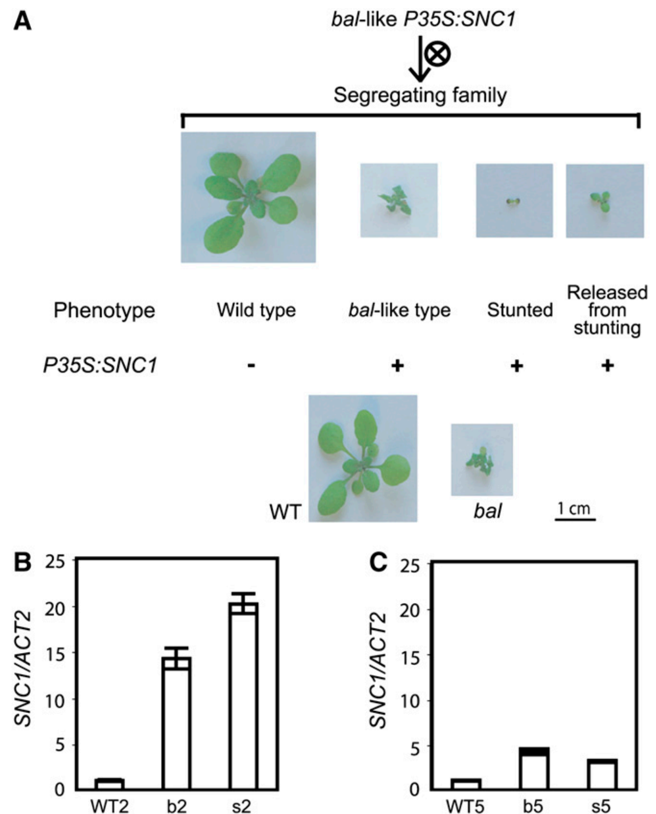

Figure 1. Transgenic Plants Overexpressing *SNC1* (*P35S:SNC1*) Display Two Distinct Morphologies.

(A) Phenotypes of segregating progeny of *bal*-like transgenic plants overexpressing *SNC1*. The presence or absence of the transgene, determined by PCR using primers for the kanamycin resistance gene, is indicated by the + and − signs, respectively. Transgenic plants with the stunting phenotype eventually developed wild-type morphology after 3 weeks. *bal*, *bal* variant.

(B) *SNC1* transcript level, relative to *ACTIN2* (*ACT2*), determined by real-time RT-PCR using 2-week-old plants. WT2, wild-type plants; b2, *bal*-like transgenic plants; s2, stunted plants.

(C) Relative *SNC1* transcript level determined by real-time RT-PCR using 5-week-old plants. WT5, wild-type plants; b5, *bal*-like transgenic plants; s5, plants released from stunting with wild-type morphology.

The genetic behavior described above suggested that the morphological consequences of *SNC1* overexpression (*bal*-like versus stunted) depend on the dosage of the *P35S:SNC1* transgene. Consistent with this explanation, the expression level of

SNC1 was higher in homozygous stunted transgenic plants compared with hemizygous *bal*-like plants (Figure 1B). We suspected that the stunting and delayed normal growth syndrome, which we observed every generation in homozygous *P35S:SNC1* transgenic plants, resulted from posttranscriptional gene silencing (PTGS), RNA silencing at the posttranscriptional level, triggered by overexpression of *SNC1*. Repeated onset of gene silencing in each generation is one feature of PTGS, and homozygosity-dependent PTGS has been observed for other *Arabidopsis* transgenes (Elmayan et al., 1998; Qin et al., 2003). Consistent with the hypothesis that the delayed normal growth in homozygous *P35S:SNC1* plants was caused by PTGS of *SNC1*, we found that the steady state expression level of *SNC1* in homozygous plants was lower compared with hemizygous plants when wild-type morphology was observed in homozygous plants (Figure 1C). We concluded that overexpression of *SNC1* induces either *bal*-like or stunted phenotypes depending on the expression level of *SNC1*.

RPP5 Locus *R* Genes Are Coordinately Upregulated at the Transcriptional Level by a Positive Feedback Mechanism Mediated through *SNC1*

Transcriptional regulation of some *R* genes in *Arabidopsis*, including *SNC1*, is governed by a positive amplification loop requiring SA accumulation (Shirano et al., 2002; Xiao et al., 2003; Yang and Hua, 2004). Because all seven *R* genes located in the *RPP5* locus, where *SNC1* is located (see Supplemental Figure 1 online), appear to be generated by local tandem duplications (Baumgarten et al., 2003; Meyers et al., 2003; Leister, 2004), we suspected that other *R* genes in the locus might also be transcriptionally activated along with *SNC1* using a conserved regulatory element in their promoters. Considering that *RPP4* was shown to be functional in resistance against fungal pathogens and *At4g16950* is the most similar *R* gene in Columbia haplotype to the *RPP5* gene in Landsberg haplotype, we focused on *RPP4* and *At4g16950* (Parker et al., 1997; Noel et al., 1999; van der Biezen et al., 2002; Meyers et al., 2003). We found that expression levels of *RPP4* and *At4g16950* were higher in *bal*, *cpr1*, and *snc1* mutants, all of which carry mutations mapping to the *RPP5* locus and show constitutive upregulation of *SNC1* (Bowling et al., 1994; Li et al., 2001; Stokes et al., 2002; Zhang et al., 2003), suggesting that *RPP5* locus *R* genes are coordinately upregulated (Figures 2A to 2C). Using transgenic plants overexpressing *SNC1*, we directly tested whether expression levels of *RPP5* locus *R* genes were transcriptionally induced by overexpression of *SNC1* and found that expression levels of both *RPP4* and *At4g16950* were elevated in *bal-like* hemizygous and homozygous stunted *P35S:SNC1* transgenic plants (Figures 3A and 3B). These data indicate that *RPP4* and *At4g16950*, as well as *SNC1*, are regulated by a transcriptional amplification mechanism mediated by SNC1 activity.

RPP5 Locus *R* Genes Can Be Coordinately Downregulated by Cosuppression in Plants Overexpressing *SNC1*

In recent years, many small RNA species in *Arabidopsis* have been identified from large-scale small RNA cDNA sequencing

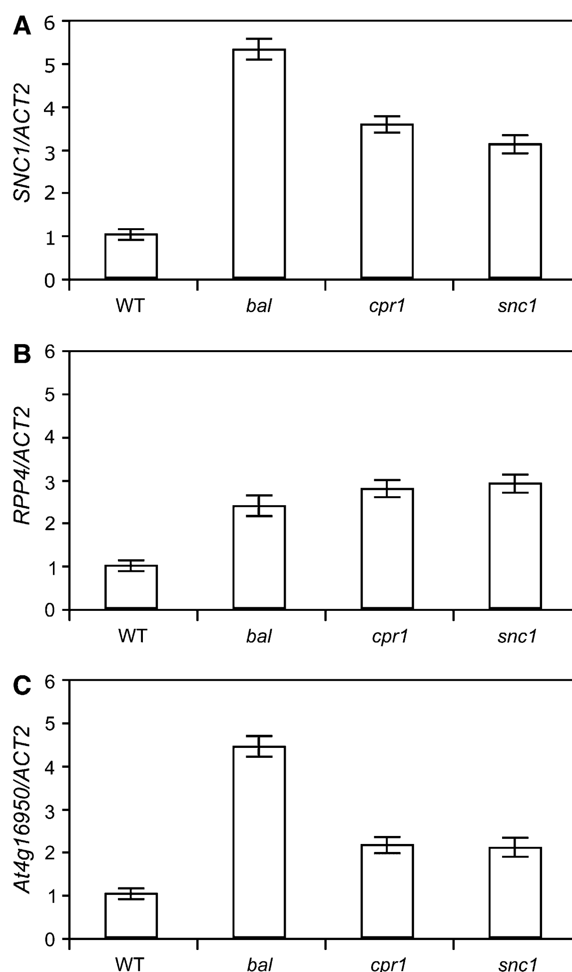

Figure 2. Steady State Expression Levels of *RPP4* and *At4g16950* Increase in Mutants That Upregulate *SNC1*.

(A) *SNC1* transcript level, relative to *ACT2*, determined by real-time RT-PCR.

(B) and **(C)** Relative transcript levels of *RPP4* and *At4g16950* determined by real-time RT-PCR. Tissue for RNA isolation was collected from 2-week-old plants.

projects, and three research groups have independently reported diverse small RNA species originating from *RPP5* locus *R* genes (Nakano et al., 2006; Rajagopalan et al., 2006; Kasschau et al., 2007). Although most of these small RNA species were identified only a single time and many of them showed perfect matches to more than one *RPP5* locus *R* gene, including *SNC1*, some *SNC1*-specific small RNA were also isolated (Figure 4A). A collective data set revealed that small RNA species in various size classes are generated from both strands of *SNC1* gene and that these small RNA species are predominantly 24 nucleotides and produced in the central part of the *SNC1* gene, downstream of the region encoding the NBS domain.

We confirmed that 21- to 24-nucleotide small RNA species, the hallmark of RNA silencing, are generated from *RPP5* locus *R* gene transcripts by performing a small RNA gel blot experiment.

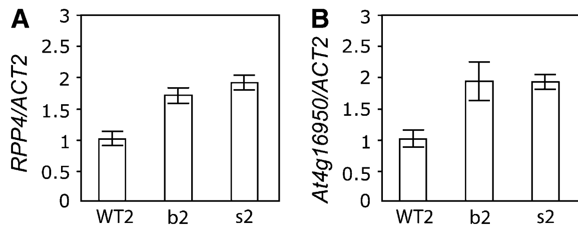

Figure 3. Expression Levels of *RPP4* and *At4g16950* Correlate with *SNC1* in *P35S:SNC1* Transgenic Plants.

RPP4 and *At4g16950* transcript levels, relative to *ACT2*, determined by real-time RT-PCR using 2-week-old plants. WT2, wild-type plants; b2, *bal*-like transgenic plants; s2, stunted plants.

Discrete 21-nucleotide small RNA species were detected in vegetative tissues using a hybridization probe corresponding to the conserved LRR domain in *RPP5* locus *R* genes (Figure 4B). In inflorescence tissue, 24-nucleotide small RNA species and 21-nucleotide species were observed, although the smaller size class was more abundant. The difference in the prevalence of the different size classes between the small RNA sequencing projects and our experiments may be due to the specific hybridization probe used in our small RNA gel blot experiment and the preferential use of inflorescence tissue in the small RNA sequencing projects.

We investigated the possibility that *SNC1* and other *RPP5* locus *R* genes are coordinately regulated by RNA silencing at the posttranscriptional level based on their nucleotide sequence similarity. We tested whether the nucleotide sequences of *SNC1* and other *RPP5* locus *R* genes are similar enough to be targeted by RNA silencing in a concerted manner, as was suggested by the presence of small RNA species that are perfect matches to more than one *R* gene in the *RPP5* locus. We reasoned that expression levels of *RPP4* and *At4g1695*0 might also be reduced in homozygous *P35S:SNC1* plants exhibiting wild-type morphology compared with those in *bal*-like hemizygotes, if *RPP5* locus *R* genes can be coordinately regulated by RNA silencing. We found that expression levels of both *RPP4* and *At4g16950* are lower in wild type–like *P35S:SNC1* homozygotes (released from stunting) than in *bal*-like hemizygotes at this developmental stage (Figures 4C and 4D). The expression level of *RPP4* is comparable in wild-type plants and *snc1 r1* mutants, which carry a null allele of *SNC1* (Zhang et al., 2003) (see Supplemental Figure 2A online), suggesting that the reduction of *RPP4* and *At4g16950* expression in normal-looking homozygous transgenic plants is not caused by downregulation of *SNC1.* We concluded that *RPP5* locus *R* genes can be coordinately suppressed by RNA silencing based on their nucleotide sequence similarity.

Antisense Transcripts Overlapping with *RPP4*, *SNC1*, and *At4g16900* Are Produced in the *RPP5* Locus

The generation and amplification of siRNA species, which can coordinately regulate homologous genes through RNA silencing, requires the formation of foldback structures or dsRNA (Vaucheret, 2006). Antisense transcripts originating from downstream transposons or genes have been implicated in the formation of dsRNA with sense transcripts from upstream genes (Aravin et al., 2001; Kashkush et al., 2003; Borsani et al., 2005; Katiyar-Agarwal et al., 2006). To identify the source of the small RNA species detected above, we focused on the genomic region around *SNC1* where three retrotransposon sequences, At *Copia4* (*At4g16870*), At *Copia49* solo long terminal repeat (LTR), and At *Gypsy2* (*At4g16910*) are located (Figure 4A). Signatures in the *Arabidopsis* massively parallel signature sequencing (MPSS) database suggested that antisense transcripts are made from *RPP4*, *SNC1*, or *At4g16900* (Nakano et al., 2006). First, we determined the positions of the 3′ ends of *RPP4*, *SNC1*, and *At4g16900* sense transcripts using rapid amplification of cDNA ends and obtained results consistent with those reported from 3′ end or full-length sequencing of cDNA clones from these *R* genes (Figure 4A) (van der Biezen et al., 2002; Castelli et al., 2004). The presence of antisense transcripts, which overlap at least 1 kb with sense transcripts, was validated using strand-specific RT-PCR for all three *R* genes (Figure 4E). Compared with wild-type plants, expression of the *RPP4* antisense transcript did not show any significant change in the *cpr1* mutant but showed a dramatic decrease in the *bal* variant. Antisense transcripts of *RPP4* were almost abolished in aphenotypic *ddm1-2* (for *decrease in DNA methylation 1-2*) mutants, suggesting that this change originated from the inbred *ddm1-2* mutant background from which the *bal* variant was derived (Kakutani et al., 1996). By contrast, a slight increase in the levels of antisense transcripts of *SNC1* and *At4g16900* was observed in the *bal* variant compared with wild-type plants or the *cpr1* mutant (Figure 4E). Therefore, it is unlikely that the downregulation of antisense transcripts is directly responsible for the upregulation of overlapping sense transcripts. Our results show that sense and antisense transcript pairs, which may form dsRNA and generate small RNA species, are produced from *RPP5* locus *R* genes.

The Steady State Expression Level of *SNC1* Is Elevated in Mutants Associated with Defects in RNA Silencing

Our findings that discrete small RNA species corresponding to the *RPP5* locus *R* gene transcripts are produced and that *RPP5* locus *R* genes can be cosuppressed led us to examine the effect of mutations affecting RNA silencing on *SNC1* expression. Small RNA species, which are loaded into AGOs and used to direct sequence-specific cleavage of target transcripts, are produced by DCLs from RNA duplexes whose formation may depend on RDR activities. In *Arabidopsis*, three out of six RDRs, all four DCLs, and four out of 10 AGOs have been characterized for their function in RNA silencing (Vaucheret, 2006; Zheng et al., 2007). Another protein that has been implicated in RNA silencing is UPF1, a conserved component in nonsense-mediated mRNA decay (NMD), which degrades mRNA transcripts with premature stop codons in eukaryotes (Behm-Ansmant and Izaurralde, 2006). The UPF1 ortholog in *Caenorhabditis elegans* is necessary for the persistence of RNA silencing (Domeier et al., 2000; Kim et al., 2005), and the *Arabidopsis* ortholog is required for RNA silencing triggered by an inverted repeat transgene (Arciga-Reyes et al., 2006). The steady state expression levels of *SNC1*

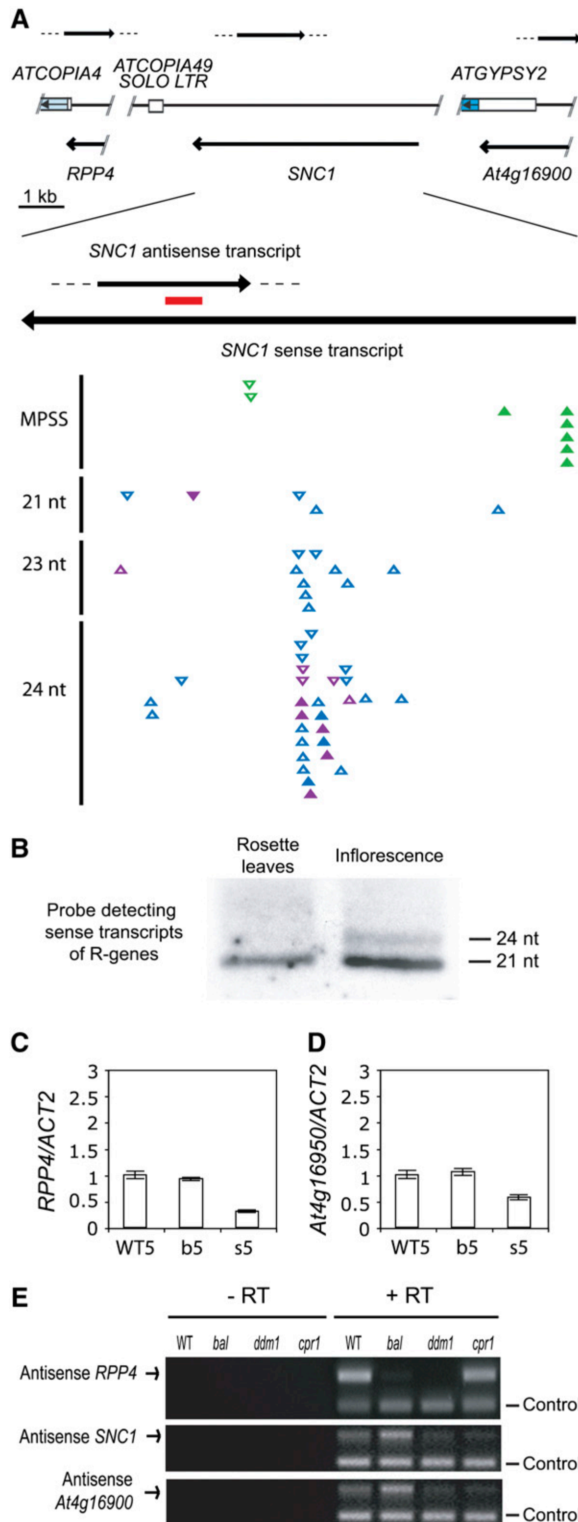

Figure 4. Small RNA Species Are Detected from Columbia *RPP5* Locus *R* Genes.

(A) Small RNA species of various size are generated from *SNC1*. Each left-to-right arrow shows the positions of antisense transcripts in the *RPP5* locus detected in **(E)**. Below the antisense transcripts, the positions of a part of At *Copia4* and At *Gypsy2* LTRs, as well as the At *Copia49* solo LTR, are indicated by rectangles. Arrows in the shaded rectangles represent the putative retrotransposon polyproteins. The coding regions of *RPP4*, *SNC1*, and *At4g16900* sense transcripts are indicated by right-to-left arrows below the representation of transposon-related sequences. The red bar in the overlapping region of *SNC1* antisense and sense transcripts shows the position of the strand-specific probe used in small RNA gel blot analysis (**[B]**; Figure 6). The 5′ and 3′ ends of the antisense transcripts, including that of *SNC1*, have not been determined and are therefore represented by dotted lines. The triangles show positions of all the reported small RNA signatures whose nucleotide sequences are perfect matches to those in *SNC1*. The small RNA species reported by Meyers/Green (MPSS), Carrington, and Bartel labs are colored in green, purple, and blue, respectively (Nakano et al., 2006; Rajagopalan et al., 2006; Kasschau et al., 2007), and are arranged by size class. *SNC1*-specific small RNA species are indicated by filled triangles, while small RNA species whose sequences are conserved among different *RPP5* locus *R* genes, including *RPP4* and *At4g16950*, are indicated by open triangles. Small RNA species originating from sense and antisense transcripts are represented by triangles pointing upward and downward, respectively. Small RNA species that were isolated multiple times are indicated by multiple triangles. Note that the small RNA species shown here are cataloged regardless of tissues or genotypes from which a given small RNA species was isolated. nt, nucleotides.

(B) RNA gel blot analysis detects small RNA species corresponding to the sense strand of the LRR region of *R* genes from the locus in rosette leaves and inflorescence tissue. The sizes of 21- and 24-nucleotide small RNA species were determined by subsequent hybridization to detect small RNA species of known size.

(C) and **(D)** Relative transcript levels of *RPP4* and *At4g16950* determined by real-time RT-PCR using 5-week-old plants. WT5, wild-type plants; b5, *bal*-like transgenic plants; s5, plants released from stunting with wild-type morphology.

(E) Antisense transcripts overlapping with *RPP4*, *SNC1*, and *At4g16900* sense transcripts are detected by strand-specific RT-PCR. −RT and + RT, first-strand cDNA library constructed without and with reverse transcriptase, respectively. *ddm1*, *ddm1-2* mutants that were inbred for two generations; control, internal loading control for multiplex RT-PCR, glyceraldehyde 3-phosphate dehydrogenase (*GAPC*).

transcripts in wild-type plants and various mutants were compared using quantitative real-time RT-PCR. As expected for pairs of overlapping sense and antisense transcripts, which can form dsRNA over a 1-kb region without RDR activity, we found that RDR1, RDR2, and RDR6 activity was dispensable for the full suppression of *SNC1* (Figure 5A). We cannot rule out the possibility that other RDRs, which were not tested here, contribute to the generation of dsRNA in the *RPP5* locus, but we note that the MPSS mRNA signatures, which originally suggested the presence of antisense transcripts in the locus, were generated using polyadenylated RNA, most likely produced by RNA polymerase II.

We found that the steady state expression level of *SNC1* transcript was elevated in some mutants defective in RNA silencing, for example, the *dcl4-4* mutant (Dunoyer et al., 2005) (Figure 5B). DCL4 is responsible for the generation of 21-nucleotide siRNA species in the trans-acting siRNA pathway as well as those produced from inverted repeat transgenes and viruses (Dunoyer et al., 2005; Gasciolli et al., 2005; Xie et al., 2005;

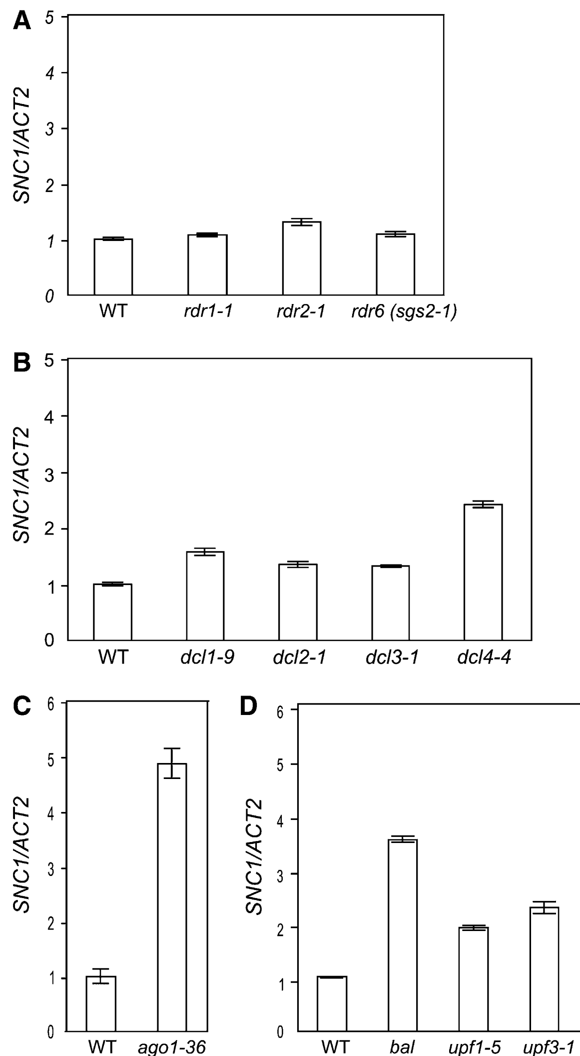

Figure 5. Steady State Expression Levels of *SNC1* Are Elevated in Mutants Affecting RNA Silencing.

(A) and **(B)** *SNC1* transcript levels, relative to *ACT2*, determined by real-time RT-PCR using plants homozygous for various *rdr* mutations and four *dcl* mutations, respectively.

(C) Relative *SNC1* transcript levels in *ago1-36* mutants compared with wild-type siblings plants.

(D) Relative *SNC1* transcript levels compared among wild-type control plants, the *bal* variant, and *upf1-5*, and *upf3-1* mutants. Plants were grown in soil except for samples used in **(C)**, where plants were grown using in vitro culture conditions. Tissue for RNA isolation was collected from 2-week-old plants.

Yoshikawa et al., 2005; Deleris et al., 2006; Fusaro et al., 2006). DCL4 was also reported to be responsible for the production of two miRNA species in *Arabidopsis* (Rajagopalan et al., 2006). No significant changes in *SNC1* expression were observed in other *dcl* mutants compared with wild-type plants (Figure 5B). Among the four AGOs with a demonstrated role in RNA silencing, AGO1 physically associates with 21-nucleotide small RNA species almost exclusively (Baumberger and Baulcombe, 2005; Qi et al., 2006). We found that the steady state expression level of *SNC1* was elevated in the *ago1-36* mutant (Baumberger and Baulcombe, 2005) (Figure 5C). However, we did not observe any significant change in *SNC1* expression level in *rpd2a-2 rpd2b-1*, *dcl3*, or *rdr2* mutants, all of which specifically affect the accumulation of 24-nucleotide small RNA (Figures 5A and 5B; see Supplemental Figure 2B online) (Onodera et al., 2005; Kasschau et al., 2007). In addition, increases in the steady state expression levels of *SNC1* were observed in hypomorphic *upf1-5* and *upf3-1* mutants, both of which are compromised for NMD (Hori and Watanabe, 2005; Arciga-Reyes et al., 2006) (Figure 5D). The activation of *SNC1* in the NMD-defective mutants might be related to the fact that alternatively spliced transcripts with premature stop codons in retained introns are produced for *SNC1*, thereby making *SNC1* a direct target of NMD (see Supplemental Figure 3 online).

Next, we examined small RNA species generated from the *RPP5* locus *R* genes in *dcl4-4*, *ago1-36*, *upf1-5*, and *upf3-1* mutants. Specifically, we monitored the abundance of 21-nucleotide antisense small RNA species corresponding to the LRR region. DCL4 and AGO1 are involved in the biogenesis and use of 21-nucleotide small RNA. The LRR region was chosen because antisense small RNA species from this conserved region are expected to negatively regulate multiple *R* genes in the *RPP5* locus. As expected, the accumulation of tasiR255 is abolished in *dcl4* and *ago1* mutants (Gasciolli et al., 2005; Adenot et al., 2006). By contrast, we did not observe any significant decrease in the levels of small RNA species corresponding to antisense transcripts of *RPP5* locus *R* genes in any of the mutants that showed increase in steady state expression of *SNC1* (Figure 6; see Supplemental Figure 4 online).

DISCUSSION

Here, we report two mechanisms that can coordinately regulate many paralogous *R* genes in the *RPP5* locus. Many *RPP5* locus *R* genes are positively regulated at the transcriptional level by a feedback amplification loop involving SNC1, and they can be cosuppressed when *SNC1* is expressed over a certain threshold. In addition, we show that the steady state expression level of *SNC1*, which can induce the activation of other *RPP5* locus *R* genes, is elevated in *dcl4-4* and *ago1-36* mutants.

RPP5 Locus *R* Genes Are Coordinately Upregulated by a Positive Feedback Mechanism Mediated by *SNC1*

It was originally proposed that *SNC1* is regulated by a positive feedback mechanism through SA accumulation (Yang and Hua, 2004). We confirmed and extended these findings by demonstrating that *RPP4* and *At4g16950*, two additional *R* genes in the locus, are also transcriptionally induced by overexpression of *SNC1* (Figure 3). Our results are consistent with the analysis of data from 128 publicly available microarray experiments that identified *Copia4* (*At4g16870*), *RPP4* (*At4g16860*), *At4g16880*, and *SNC1* (*At4g16890*) as one group of 226 highly coexpressed neighboring genes in *Arabidopsis* (Zhan et al., 2006). Our data also show that another *R* gene in the locus, *At4g16950*, is co-regulated with *RPP4* and *SNC1* when *SNC1* is overexpressed.

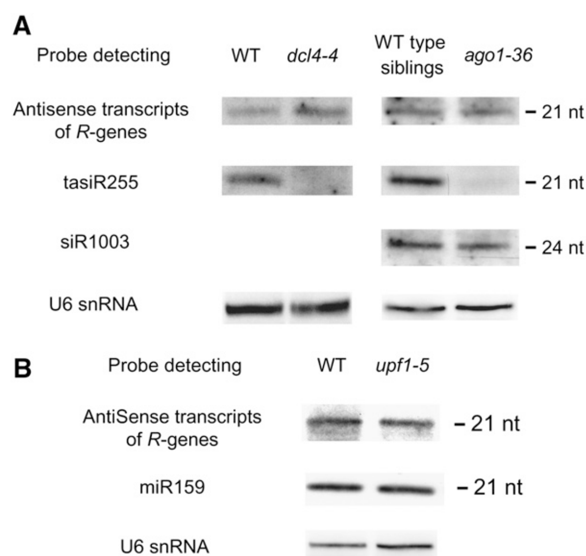

Figure 6. Accumulation of Small RNA Species Originating from Antisense Transcripts of *RPP5* Locus *R* Genes Does Not Significantly Decrease in Mutants with an Elevated *SNC1* Expression Level.

Small RNA gel blot analysis was used to detect the RNA species indicated on the left. Results shown here were obtained from a single membrane by sequential hybridization after an extensive washing. The sizes of 21- and 24-nucleotide small RNA species were determined by subsequent hybridization to detect small RNA species of known size. Note that wild-type siblings and *ago1-36* mutants were grown under in vitro culture conditions. WT siblings, siblings of *ago1-36* with wild-type morphology (i.e., wild-type plants and heterozygous *ago1-36* mutants). Tissue for RNA isolation was collected from 2-week-old plants. Similar results were found in at least two independent biological replicates of these hybridization experiments.

Even though we propose that *RPP4* and *At4g16950* are directly activated through SA accumulation resulting from SNC1 activation, we cannot rule out the possibility that coordinated upregulation of *RPP4*, *SNC1*, and *At4g16950* resulted indirectly from transcriptional activation of *SNC1*, which could change the local chromatin structure of the locus. Activation of a number of related *R* genes that function in defense signaling pathways via SA accumulation may provide plants a broader spectrum of resistance to closely related pathogens.

SNC1 and Other *RPP5* Locus *R* Genes Can Be Cosuppressed

Beginning with the identification of small RNA species showing perfect matches to many *R* genes in the *RPP5* locus, we showed that several *R* genes in the locus can be cosuppressed (Figure 4). Overexpression of *SNC1*, which constitutively activates defense signaling, reduces fitness (Heidel et al., 2004; van Hulten et al., 2006). RNA silencing might play an important role in minimizing the fitness cost associated with excessive *SNC1* expression. It is possible that RNA silencing machinery is used to quickly remove *SNC1* transcripts when *SNC1* expression level reaches a certain threshold level that cannot be tolerated or after activation by pathogen challenge. Alternatively, RNA silencing may allow the evolution of *RPP5* locus *R* genes while minimizing the possible deleterious effect caused by the recombination or multiplication of these *R* genes, as suggested for other rapidly expanding gene families in *Arabidopsis* (Howell et al., 2007).

SNC1 Is Activated When DCL4 and AGO1 Are Compromised

Our results show that *SNC1*, overexpression of which induces upregulation of other *RPP5* locus *R* genes (Figure 3), is activated when two components in RNA silencing (DCL4 and AGO1) are compromised (Figures 5B and 5C). DCL4 is responsible for the production of 21-nucleotide small RNA species from various sources (Gasciolli et al., 2005; Xie et al., 2005; Yoshikawa et al., 2005; Deleris et al., 2006; Fusaro et al., 2006), and AGO1 is almost exclusively associated with 21-nucleotide small RNA species (Baumberger and Baulcombe, 2005; Qi et al., 2006). We detected convergent, overlapping sense and antisense transcripts along with 21-nucleotide small RNA species corresponding to several *R* genes in the *RPP5* locus. Moreover, the genetic requirement of DCL4 and AGO1 for suppression of *SNC1* expression is consistent with our detection of 21-nucleotide small RNA species from these *R* genes. The simplest model predicts that the abundance of 21-nucleotide RNA species corresponding to the antisense strand of the *R* genes would decrease in *dcl4-4* and *ago1-36* mutants. However, we did not observe an inverse correlation between the abundance of these small RNA species and their putative targets in either *dcl4-4* or *ago1-36* mutants (Figure 6). One possibility is that the probe used in our gel blot hybridization experiments did not detect the functional small RNA species responsible for downregulating *R* gene transcripts. The probe was chosen to recognize the conserved LRR region corresponding to the antisense 21-nucleotide small RNA species (Figure 4B), which are the best candidates for coordinate regulation of *RPP5* locus *R* genes. An alternative hypothesis is that the 21-nucleotide small RNA species detected in Figure 6 are responsible for targeting *R* gene turnover and are only transiently reduced in the *dcl4-4* and *ago1-36* mutants. The result in Figure 6 may reflect a steady state equilibrium where a wild-type level of small RNA species, which are produced and stabilized by other DCLs and AGOs, is now balanced against a higher concentration of *R* gene transcript targets. A third possibility is that the activation of the *RPP5* locus *R* genes in *dcl4-4* and *ago1-36* mutant backgrounds is indirect, caused by an RNA silencing pathway defect affecting one or more upstream components that transcriptionally activate *SNC1* (Yang and Hua, 2004).

Whether *SNC1* is directly or indirectly activated by release of RNA silencing, our results show that *SNC1* can be upregulated if some components in RNA silencing are functionally compromised. Therefore, RNA silencing may function as a sensor to detect any interference in RNA silencing machinery by pathogens, leading to the initiation of defense responses that are mediated through the activation of *SNC1* and other *RPP5* locus *R* genes. Consistent with this hypothesis, an increase in steady state expression level of *SNC1* was observed from transgenic plants overexpressing a viral suppressor of RNA silencing suppressor, P1/Helper Component-Protease (P1/HC-Pro) (see Supplemental Figure 5 online). Furthermore, DCL4, which is required

for the full suppression of *SNC1*, is the primary DCL involved in viral defense and is a target of p38, another viral RNA silencing suppressor (Deleris et al., 2006). Several RNA silencing pathways, which require different combinations of RDRs, DCLs, AGOs, and other components, have been identified in *Arabidopsis* (Brodersen and Voinnet, 2006; Vaucheret, 2006). However, the genetic requirement of DCL4 and AGO1 for the suppression of *SNC1* is different from the requirements defined for other pathways regulating endogenous targets. For example, DCL4 is required for the production of 21-nucleotide trans-acting siRNA, but this pathway also requires the concerted action of DCL1, RDR6 (SGS2), and SGS3 to generate dsRNA (Gasciolli et al., 2005). Our results show that loss of DCL1, RDR6, or SGS3 does not affect steady state expression level of *SNC1* significantly (Figures 5A and 5B; see Supplemental Figure 6A online). Instead, the genetic requirement to suppress *SNC1* expression is similar to that expected for the RNA silencing pathway targeting inverted repeat transgenes (IR-PTGS pathway), which has been widely used to knock down specific genes in transgenic plants (Wesley et al., 2001). The IR-PTGS pathway requires DCL4 and AGO1 (Dunoyer et al., 2005, 2007), but RDR6 and HEN1 are dispensable (Boutet et al., 2003; Li et al., 2005), as is the case for the suppression of *SNC1* (Figure 5A; see Supplemental Figure 6B online). The overlap of genetic requirements between the IR-PTGS pathway and suppression of *SNC1* suggests that *SNC1* itself or an upstream component in the signaling cascade that results in the activation of *SNC1* is one natural target of the IR-PTGS pathway.

Accumulating evidence shows that RNA silencing is used to regulate endogenous genes more extensively than previously thought. For example, posttranscriptional control of the *chalcone synthase* gene family has been reported for naturally generated variants of soybean (*Glycine max*), maize (*Zea mays*), and petunia (*Petunia hybrida*) (Senda et al., 2004; Della Vedova et al., 2005; Koseki et al., 2005). Many clustered paralogous genes in plant genomes might generate inverted repeat transcripts or antisense transcripts, thereby providing an opportunity for coordinated suppression of these genes by RNA silencing. Our results show that RNA silencing can be used to restrict the fitness cost associated with the constitutive activation of defense signaling involving *RPP5* locus *R* genes, which are controlled by a feedback amplification loop. In addition, our results demonstrate that defense mechanisms can be activated in response to pathogen attack that disturbs the RNA silencing machinery.

METHODS

Plants and Growth Conditions

All plant genotypes used in this study were in the Columbia background and inbred a few generations except hypomorphic *dcl1-9* mutation that causes female sterility. Our *dcl1-9* strain carrying the Columbia *RPP5* locus was generated by crossing a Landsberg strain carrying *dcl1-9* (Jacobsen et al., 1999) to wild-type plants in the Columbia background and subsequent segregation. We previously described *bal* and *cpr1* as well as the *ddm1-2* mutant (Kakutani et al., 1996; Stokes et al., 2002; Stokes and Richards, 2002). *rdr1-1*, *rdr2-1* (Xie et al., 2004), *rdr6 (sgs2-1)* (Elmayan et al., 1998), *dcl2-1*, *dcl3-1* (Xie et al., 2004), *dcl4-4* (Dunoyer et al., 2005), *snc1 r1* (Zhang et al., 2003), *hen1-5* (Vazquez et al., 2004a), *rpd2a-2 rpd2b-1* mutants (Onodera et al., 2005), and transgenic plants overexpressing the P1/HC-Pro viral suppressor (Chapman et al., 2004; Mlotshwa et al., 2005) were kindly provided by James C. Carrington (*dcl2-1*, *dcl3-1*, *rdr1-1*, *rdr2-1*, and *P1/HC-Pro*), Xin Li (*snc1 r1*), Craig S. Pikaard (*rpd2a rpd2b*), Vicki B. Vance (*P1/HC-Pro*), Hervé Vaucheret (*rdr6* and *hen1-5*), and Olivier Voinnet (*dcl4-4*). In addition, *upf1-5* (Arciga-Reyes et al., 2006), *upf3-1* (Hori and Watanabe, 2005), and *sgs3-13* (Peragine et al., 2004) mutants were obtained from the ABRC (Alonso et al., 2003).

All plants except *ago1* and sibling plants were grown on soil in growth chambers under long-day conditions (16 h light) as described previously (Stokes et al., 2002). Homozygous *ago1-36* mutants (Baumberger and Baulcombe, 2005) and segregating siblings were grown on solid media (half-strength Murashige and Skoog basal salt mixture [Sigma-Aldrich], 1% sucrose, and 4% Agarasel [Sigma-Aldrich]) in growth chambers under long-day conditions (16 h light).

Nucleic Acid Isolation

Total RNA and low molecular weight–enriched RNA were isolated from aerial parts of either 2- or 5-week-old plants using TRIzol reagent (Invitrogen) and the mirVana miRNA isolation kit (Ambion), respectively, following the manufacturers' protocols. Genomic DNA for genotyping was isolated by the urea lysis miniprep protocol (Cocciolone and Cone, 1993).

Expression Analysis

To analyze the expression level of genes by real-time RT-PCR, first-strand cDNA libraries were constructed with total RNA samples using the SMART RACE cDNA amplification kit (Clontech), after treating total RNA with RQ1 RNase-free DNase I (Promega). Antisense strand-specific libraries were constructed with GAPCR and balANTI primers, instead of the 3′ RACE CDS primer A in the kit. Nucleotide sequences of the primers and Taqman probes used in our experiments are listed in Supplemental Table 1 online. For quantitative real-time PCR, the Applied Biosystems 7500 Real Time PCR system and Taqman MGB probe were used. Triplicate PCR reactions were used to generate error bars in every experiment, and at least two biologically independent experiments were performed for every case to confirm the results. For multiplex RT-PCR, the ratio between GAPC primers with or without dideoxy modification was empirically determined (Kerschen et al., 2004).

Small RNA gel blot analysis was performed as previously described with minor modifications (Onodera et al., 2005). In brief, a denaturing 15% (v/v) acrylamide gel was used to separate low-molecular RNA, and PefectHyb Plus hybridization buffer (Sigma-Aldrich) was used. Strand-specific probes were made with the mirVana miRNA probe construction kit (Ambion) using oligonucleotides or PCR products with T7 polymerase binding sites. After hybridization at 40°C, membranes were washed as follows at the same temperature: 15 min in 2× SSC and 0.1% SDS, 15 min in 1× SSC and 0.1% SDS, and 15 min in 0.5× SSC and 0.1% SDS. Nucleotide sequences for oligonucleotides or primers used in probe construction are in Supplemental Table 1 online.

Accession Numbers

The following loci were studied in this article: *At4g16860* (*RPP4*), *At4g16870* (*Copia4*), *At4g16890* (*SNC1*), *At4g16900*, *At4g16910* (*Gypsy2*), and *At4g16950*.

Supplemental Data

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

Supplemental Figure 1. Organization of *R* Genes Located in *RPP5* Locus of the Columbia Haplotype.

Supplemental Figure 2. The Relative Quantity of *RPP4* in the *snc1 r1* Mutant and That of *SNC1* in the *rpd2a-2 rpd2b-1* Double Mutant Are Not Changed Compared with Wild-Type Plants.

Supplemental Figure 3. *SNC1* Transcripts Are Alternatively Spliced to Produce Transcripts with or without Introns.

Supplemental Figure 4. No Significant Difference in the Accumulation of Small RNA Species Originating from Antisense Transcripts of *RPP5* Locus *R* Genes Was Detected between Wild-Type Plants and *upf3-1* Homozygotes.

Supplemental Figure 5. Expression Levels of *SNC1* and *RPP4* Are Elevated in Transgenic Plants Overexpressing a Viral Suppressor of RNA Silencing, P1/HC-Pro.

Supplemental Figure 6. The Relative Quantity of *SNC1* Is Comparable in the *sgs3-13* or *hen1-5* Mutant Compared with Wild-Type Plants.

Supplemental Table 1. Nucleotide Sequences for Oligonucleotides or Primers Used.

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