The disease resistance protein SNC1 represses the biogenesis of microRNAs and phased siRNAs

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Plants evolved an array of disease resistance genes (R genes) to fight pathogens. In the absence of pathogen infection, NBS-LRR genes, which comprise a major subfamily of R genes, are suppressed by a small RNA cascade involving microRNAs (miRNAs) that trigger the biogenesis of phased siRNAs (phasiRNAs) from R gene transcripts. However, whether or how R genes influence small RNA biogenesis is unknown. In this study, we isolate a mutant with global defects in the biogenesis of miRNAs and phasiRNAs in Arabidopsis thaliana and trace the defects to the over accumulation and nuclear localization of an R protein SNC1. We show that nuclear SNC1 represses the transcription of miRNA and phasiRNA loci, probably through the transcriptional corepressor TPR1. Intriguingly, nuclear SNC1 reduces the accumulation of phasiRNAs from three source R genes and concomitantly, the expression of a majority of the ~170 R genes is up-regulated. Taken together, this study suggests an R gene-miRNA-phasiRNA regulatory module that amplifies plant immune responses.
Plants often suffer from attacks by various pathogens and have evolved a series of defense mechanisms. One important mechanism is Resistance (R) gene-mediated disease resistance. R proteins are activated upon pathogen infection and cause local programmed cell death named hypersensitive response (HR). Local HR often leads to systemic and broad-spectrum resistance to pathogens called systemically acquired resistance.

R genes are classified into at least five groups according to domain composition. Among them, the NBS-LRR protein coding transcripts. A cleavage fragment from these siRNAs). PhasiRNAs are a class of small RNAs that is widespread in plant genomes, usually numbering in hundreds. The genome of Arabidopsis thaliana contains approximately 170 NBS-LRR genes.

While R genes confer resistance to pathogens, their constitutive expression is detrimental to plant growth such that their expression must be tightly regulated. Some NBS-LRR genes are silenced by microRNAs (miRNAs), a notable example is the tobacco N gene that confers resistance toward tobacco mosaic virus (TMV). But repression of R gene expression by small RNAs is not limited to individual miRNA–R gene interactions and entails global effects mediated by phased siRNAs (phasiRNAs). PhasiRNAs are a class of small RNAs that is widespread in the plant kingdom. PhasiRNA biogenesis is triggered by miRNA-guided cleavage of noncoding transcripts or certain protein coding transcripts. A cleavage fragment from these transcripts becomes the source of phasiRNAs. In Arabidopsis, eight noncoding TAS loci generate phasiRNAs, and these small RNAs were originally named trans-acting small interfering RNAs (tasiRNAs) as they regulate targets in trans. Emerging evidence indicates that plants utilize specific miRNAs to target some NBS-LRR genes and trigger the production of phasiRNAs from these transcripts. The phasiRNAs are thought to regulate NBS-LRR genes either in cis or in trans, forming a self-reinforcing regulatory network that globally suppresses R gene expression.

In Arabidopsis, miR472 and miR825 target two NBS-LRR genes and trigger the production of phasiRNAs from these transcripts.

Given that R genes are globally repressed by a miRNA–phasiRNA cascade, how do plants overcome this repression to induce and maintain R gene expression during defense? Do R genes also influence small RNA biogenesis? In a genetic screen designed to uncover players of miRNA biogenesis, we isolated a mutant with global defects in miRNA biogenesis. Rather unexpectedly, the defects in miRNA biogenesis in this mutant were traced to the over accumulation and nuclear localization of the R gene product SNC1. We showed that nuclear SNC1 likely inhibited miRNA biogenesis by repressing the transcription of MIR genes together with its interacting protein TPR1, a transcriptional corepressor. SNC1 and TPR1 also repressed the accumulation of phasiRNAs from three R genes and led to a global induction of the R family. This work revealed a mechanism whereby an activated R protein may relieve the global repression of R genes by small RNAs to enable defense. While the previously known miRNA–phasiRNA–R genes module represses R gene expression in the absence of pathogens, the R gene–miRNA–phasiRNA module discovered in this study likely enables R gene expression during defense.

### Results

Isolation of a mutant with reduced abundance of miRNAs. An ethylmethane sulfonate (EMS) mutagenesis screen was performed with the Arabidopsis line pSUC2::amiR-SUL, which will be referred to as amiR-SUL thereafter, to identify genes required for miRNA biogenesis and/or activity. In this amiR-SUL line, an artificial miRNA is expressed in the vasculature and silences the endogenous chlorophyll biosynthesis gene CHLORINA42 (also known as SULFUR, SUL) in mesophyll cells to result in vein-centered leaf bleaching. We isolated a suppressor mutant (amiR-SUL-sup-B65) with reduced vein-centered bleaching, a phenotype implying compromised amiR-SUL activity (Fig. 1a, b). In addition to reduced leaf bleaching, amiR-SUL-sup-B65 showed pleiotropic phenotypes, including dwarfism and twisted leaves (Fig. 1a, b). The amiR-SUL-sup-B65 mutant was backcrossed with the parental amiR-SUL line, and plants resembling amiR-SUL-sup-B65 in morphology (reduced plant size and reduced leaf bleaching) constituted 1/16 of the F2 population (20 out of 337 F2 plants); this segregation ratio is consistent with the phenotype being caused by two unlinked, recessive mutations ($\chi^2 = 0.05; P = 0.822$; Table 1). The amiR-SUL-sup-B65 line was also crossed with wild type (Col) to remove the amiR-SUL transgene. Plants with morphology (reduced plant size) similar to amiR-SUL-sup-B65 but without the amiR-SUL transgene were recovered and will be referred to as sup-B65 (Supplementary Fig. 1a).

The reduced leaf bleaching in amiR-SUL-sup-B65 could be caused by lower levels of amiR-SUL. Indeed, northern blotting showed that the levels of amiR-SUL were reduced to approximately 70% of wild type levels (Figs. 1d and 2b). The expression of SUL was anticorrelated with that of amiR-SUL—the levels of both SUL mRNA (Fig. 2c) and protein (Fig. 2d) were increased in amiR-SUL-sup-B65 as compared to amiR-SUL.

In order to investigate whether this mutant had a general defect in miRNA biogenesis, we performed northern blotting to examine endogenous miRNAs. The levels of all tested miRNAs (miR156, miR164, miR167, miR319, and miR390) were reduced by 10–30% in amiR-SUL-sup-B65 relative to amiR-SUL (Fig. 1d). To obtain a global perspective on miRNAs, we performed small RNA sequencing from 15-day-old seedlings of amiR-SUL and amiR-SUL-sup-B65 in three biological replicates. A global reduction in miRNA accumulation was observed in amiR-SUL-sup-B65 (Fig. 1e and Supplementary Data 1). Therefore, the amiR-SUL-sup-B65 mutant is partially defective in miRNA biogenesis.

### Mutations in CPR1 and ABA1 Underlie the Mutant Phenotypes

Whole genome resequencing was performed to identify the mutations in amiR-SUL-sup-B65. The amiR-SUL-sup-B65 mutant was backcrossed with the parental amiR-SUL line, and F2 plants exhibiting the phenotypes of amiR-SUL-sup-B65 (reduced plant size and reduced leaf bleaching) were pooled for resequencing. Two candidate mutated sites were identified, one being a transition of G to A, which results in the substitution of Gly182 for Glu, in CPR1 (At4g12560) (Fig. 1c) and the other being a transition of C to T, which leads to the substitution of Pro125 for Leu, in ABA1 (At5g63070) (Fig. 1c). These two mutations were named cpr1–4 and aba1–8, respectively. We first tested the linkage of these two mutations with the amiR-SUL-sup-B65 phenotype (dwarf morphology and reduced area of vein-centered bleaching). In the F2 progeny of the backcross with the parental amiR-SUL line, 72 plants with the amiR-SUL-sup-B65 phenotype were genotyped for cpr1–4 and aba1–8. All 72 plants were homozygous for both mutations, indicating that the visible phenotype of amiR-SUL-sup-B65 was linked to cpr1–4 and aba1–8. From the same F2 population, we also genotyped plants that do not have the amiR-SUL-sup-B65 phenotype and identified homozygous plants containing only the cpr1–4 or the aba1–8 mutation. The aba1–8 mutation had no effect on the leaf bleaching phenotype, and the cpr1–4 mutation had a weak effect, as reflected by the “greener” look of the amiR-SUL-cpr1–4 plants in comparison to amiR-SUL plants (Fig. 1a). Neither mutation by itself caused any drastic changes in plant size (Fig. 1a).
We identified cpr1–4 and aba1–8 single mutants as well as the cpr1-4 aba1–8 double mutant without the amiR-SUL transgene in the F2 progeny of a cross between amiR-SUL sup-B65 and Col (Supplementary Fig. 1a). The two single mutants were larger than the double mutant (Supplementary Fig. 1a). To confirm that the combination of mutations in CPR1 and ABA1 leads to a drastic reduction in plant size, we obtained two T-DNA insertion lines, SALK_045148 (cpr1–3) and SALK_059469 (which we named aba1–7), and generated the double mutant. The cpr1–3 aba1–7 double mutant, but not either single mutant, had drastically reduced plant size resembling cpr1–4 aba1–8 (Supplementary Fig. 1b).

We performed complementation tests to confirm that the reduced plant size in cpr1–4 aba1–8 and the reduced leaf bleaching in amiR-SUL sup-B65 were due to mutations in both CPR1 and ABA1. Both cpr1–4 aba1–8 and amiR-SUL sup-B65 plants were transformed with pCPR1:CPR1-eYFP or pABA1:ABA1-GFP. Either transgene fully rescued the morphological defects of amiR-SUL sup-B65 (Fig. 2a) and cpr1–4 aba1–8 (Supplementary Fig. 1c), indicating that only the combination of cpr1 and aba1 mutations led to drastically reduced plant size. The reduced leaf bleaching in amiR-SUL sup-B65 was also rescued by either transgene (Fig. 2a). Consistently, either transgene restored the levels of amiR-SUL (Fig. 2b), as well as the levels of SUL RNA.
(Fig. 2c) and SUL protein (Fig. 2d), to wild-type levels. Moreover, either transgene rescued the defect in miR164 accumulation in the amiR-SUL sup-B65 mutant (Fig. 2b). Therefore, the miRNA biogenesis and plant size defects of amiR-SUL sup-B65 were attributable to a combination of cpr1–4 and aba1–8. For simplicity, we thereafter refer to the double mutant cpr1–4 aba1–8 as cpr1 aba1.

**MIR promoter activities are reduced in cpr1 aba1.** We sought to determine how miRNA biogenesis is affected by mutations in CPR1 and ABA1. We first tested whether the levels of endogenous miRNAs are affected by cpr1 and aba1 mutations when the amiR-SUL transgene is not present. We determined the levels of miR159, miR164, miR319, and miR394 in 15-day-old seedlings of cpr1 aba1 and wild type by northern blotting. All of them showed reduced levels in cpr1 aba1.

**Table 1 Segregation analysis in the F2 population**

| F2 backcross (female × male) | Number of plants with amiR-SUL sup-B65 phenotype | Number of total F2 plants | Plants with amiR-SUL sup-B65 phenotype/total (expected) | $\chi^2$ (P value) for 1:16 |
|-----------------------------|-----------------------------------------------|---------------------------|--------------------------------------------------------|---------------------------|
| amiR-SUL × amiR-SUL sup-B65 | 20                                            | 337                       | 5.93% (6.25%)                                           | 0.05 (0.822)              |

**Fig. 2** Simultaneous mutations in CPR1 and ABA1 cause the phenotypes of amiR-SUL sup-B65. **a** Plants of the indicated genotypes. amiR-SUL sup-B65 plants were transformed with pCPR1:CPR1-eYFP or pABA1:ABA1-GFP. Either transgene fully rescued the morphological defects of amiR-SUL sup-B65. Bar = 1 cm. **b** Northern blots to determine the abundance of amiR-SUL and an endogenous miRNA in plants of the indicated genotypes. Two independent transgenic lines of pCPR1:CPR1-eYFP and pABA1:ABA1-GFP were used. U6 served as the internal control. The numbers indicate relative abundance of the miRNAs. **c** Relative transcript levels of the amiR-SUL target gene SUL in the indicated genotypes as determined by quantitative RT-PCR. The levels of SUL transcript in amiR-SUL sup-B65 were restored to those in amiR-SUL by either the pCPR1:CPR1-eYFP or pABA1:ABA1-GFP transgene. Error bars represent standard deviations calculated from three biological replicates. **d** Western blots to determine SUL protein levels in plants of the same genotypes as in **b**. GAPDH served as a loading control. SUL protein levels were elevated in amiR-SUL sup-B65 but restored by either pCPR1:CPR1-eYFP or pABA1:ABA1-GFP in two independent transgenic lines. Source data are provided as a Source Data file.
a modest reduction in cpr1 aba1 (Fig. 3a). Given that mature miRNAs are processed from pri-miRNAs (primary miRNAs), we next performed real-time RT-PCR to examine the levels of eleven pri-miRNAs in Col and cpr1 aba1. The levels of these pri-miRNAs were decreased in cpr1 aba1 as compared to Col except for pri-miR393b (Fig. 3b). The reduced levels of pri-miRNAs may result from reduced transcription of MIR genes or enhanced degradation of pri-miRNAs. We tested whether the reduction in pri-miRNA levels is attributable to impaired MIR promoter activity. The cpr1 aba1 double mutant was crossed with a transgenic line in which a GUS reporter gene driven by the MIR167a promoter (pMIR167a:GUS) is inserted into a single locus12,24. In the F3 generation, we obtained cpr1 aba1 plants containing homozygous pMIR167a:GUS. GUS activity was visibly lower in cpr1 aba1 than in Col as revealed by GUS staining (Fig. 3c). Real-time RT-PCR analysis confirmed that the cpr1 aba1 double mutant had lower GUS transcript levels than wild type (Fig. 3d). Therefore, defects in miRNA biogenesis in cpr1 aba1 are likely due to reduced transcription of MIR genes.

** Derepression of SNC1 suppresses miRNA biogenesis. **CPR1 encodes an F-Box protein that functions as a negative regulator of the R protein SNC1 likely through SCF (Skp1-cullin-F-box)-mediated protein degradation22,25. Loss of CPR1 function leads to increased SNC1 protein levels and constitutive defense responses25. ABA1 encodes a zeaxanthin epoxidase that functions in the first step of the biosynthesis of the abiotic stress hormone abscisic acid (ABA)26. While ABA is well known for its role in plants' resistance to abiotic stresses27, a recent study revealed that ABA deficiency promotes the activity and nuclear localization of SNC1 and other R proteins such as RPS428. Therefore, it is reasonable to speculate that SNC1 underlies the amiR-SUL suppressor phenotype of amiR-SUL cpr1 aba1.

To test this hypothesis, we first crossed amiR-SUL cpr1 aba1 with snc1–1, a loss-of-function mutant of SNC129. In contrast to amiR-SUL cpr1 aba1, amiR-SUL cpr1 aba1 snc1–1 had the same phenotype as amiR-SUL, in terms of both plant size and leaf bleaching area (Fig. 4a). Thus, loss of function in SNC1 fully rescued the amiR-SUL cpr1 aba1 phenotype, indicating that CPR1 and ABA1 promote amiR-SUL accumulation by suppressing SNC1 expression. Consistent with this, the SNC1 protein abundance was drastically elevated in amiR-SUL cpr1 aba1 relative to amiR-SUL (Fig. 4c).

The gain-of-function mutant snc1–1 exhibits derepressed SNC1 expression and constitutive defense responses30. We introduced snc1–1 into the amiR-SUL background to evaluate its effects on amiR-SUL. As expected, the abundance of the SNC1 protein was elevated in amiR-SUL snc1–1 relative to amiR-SUL (Fig. 4d). The amiR-SUL snc1–1 plants were smaller than amiR-SUL plants and showed a mild reduction in leaf bleaching (Fig. 4b), but the phenotypes were much weaker than those of amiR-SUL cpr1 aba1 (Fig. 4a). We performed small RNA sequencing of amiR-SUL and amiR-SUL snc1–1 to determine the global profiles of miRNAs. Although some miRNAs were reduced in abundance in amiR-SUL snc1–1 as compared to amiR-SUL (Supplementary Data 2), no significant global reduction in miRNA levels was observed (Supplementary Fig. 2). On the other hand, amiR-SUL cpr1 aba1 showed a global reduction in miRNA accumulation as compared to either amiR-SUL or amiR-SUL snc1–1 (Supplementary Fig. 2). Given that aba1 mutations are expected to enhance the nuclear localization of SNC1, we reasoned that aba1 mutations would enhance the snc1–1 phenotype. Indeed, amiR-SUL snc1–1 aba1–8

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**Fig. 3** MIR promoter activities are reduced in cpr1 aba1. **a** Northern blots for four miRNAs in 15-day-old seedlings of wild type (Col) and cpr1 aba1. U6 served as the loading control. The numbers indicate relative abundance. **b** The levels of eleven pri-miRNAs in wild type (Col) and cpr1 aba1 as determined by quantitative RT-PCR. Error bars represent standard deviation calculated from three biological replicates (*Student’s t test: P < 0.05). **c** GUS staining of pMIR167a:GUS and pMIR167a:GUS cpr1 aba1 seedlings. Note that the pMIR167a:GUS transgene was at a single locus and was homozygous in the two lines. The staining was visibly lower in pMIR167a:GUS cpr1 aba1. Bar = 5 mm. **d** GUS transcript levels as determined by quantitative RT-PCR analysis of the genotypes in c. Error bars represent standard deviations calculated from three biological replicates (*Student’s t test: P < 0.05). Source data are provided as a Source Data file.
plants had stronger phenotypes than *amiR-SUL snc1-1* in both plant size and leaf bleaching (Fig. 4a, b). The levels of the SNC1 protein were not drastically different between *amiR-SUL snc1-1 aba1-8* and *amiR-SUL snc1-1* (Fig. 4d), consistent with the hypothesis that *aba1-8* promoted the nuclear localization rather than the abundance of the SNC1 protein.

To further evaluate the effects of *snc1-11, snc1-1*, and *snc1-1 aba1-8* mutations on endogenous miRNAs, we performed northern blotting in these genotypes for miR156, miR159, miR164, and miR319. These miRNAs were unaffected in *snc1-11*, but all of them showed a 30–40% reduction in *snc1-1 aba1-8*, and two of them (miR156 and miR164) were also reduced in *snc1-1* (Fig. 4c). We performed real-time RT-PCR to examine the levels of five pri-miRNAs (pri-miR156a, pri-miR159b, pri-miR164b, pri-miR166a, and pri-miR319b) in these genotypes. The pri-miRNAs were unaffected in *snc1-11*, while all of them were decreased by 35–60% in *snc1-1 aba1-8*. Three pri-miRNAs showed a significant reduction in *snc1-1* (Fig. 4e). Thus, the *aba1-8* mutation enhanced *snc1-1* in terms of its miRNA biogenesis defects. Taken together, these results suggested that the over accumulation and nuclear localization of SNC1 decrease the abundance of miRNAs by repressing the transcription of MIR genes.

**Induced SNC1 nuclear accumulation inhibits MIR transcription.** Previous studies revealed a correlation between the nuclear abundance of SNC1 and its activity in disease resistance. Our genetic studies led to the hypothesis that nuclear SNC1 represses...
miRNA biogenesis. To test this hypothesis, we sought to induce the nuclear accumulation of SNC1 at the transcriptional level through a β-estriadiol-inducible promoter. A SNC1–NLS–GFP fusion was generated by inserting a nuclear localization signal (NLS) from the simian virus (SV40) large T-antigen between SNC1 and GFP, while NLS–GFP and SNC1–GFP were also generated for comparison.

NLS–GFP, SNC1–GFP, and SNC1–NLS–GFP transgenic seedlings were treated with DMSO or 50 μM β-estriadiol (dissolved in DMSO). GFP fluorescence was detectable in root cells of β-estriadiol-treated but not mock-treated plants (Fig. 5a). As expected, NLS–GFP was largely nuclear localized (Fig. 5a). SNC1–GFP and SNC1–NLS–GFP signals were present in both the nucleus and the cytoplasm, with the latter being more enriched in the nucleus (Fig. 5a). RNA was extracted from these plants and real-time RT-PCR was performed to determine the levels of five pri-miRNAs (pri-miR159a, pri-miR159b, pri-miR164b, primiR166a, and pri-miR167a). For SNC1–GFP, the levels of all five pri-miRNAs were reduced by 15–40% in β-estriadiol-treated plants relative to mock-treated plants. For SNC1–NLS–GFP, the levels of all five pri-miRNAs were reduced by 35–57% in β-estriadiol-treated plants relative to mock-treated plants. For SNC1–GFP, the levels of four of the five pri-miRNAs were reduced by 15–40% in β-estriadiol-treated plants relative to mock-treated plants. For SNC1–NLS–GFP and SNC1–GFP plants, three of the five pri-miRNAs were more severely affected in β-estriadiol-treated SNC1–NLS–GFP plants than β-estriadiol-treated SNC1–GFP plants. Thus, the nuclear accumulation of SNC1 represses the biogenesis of some miRNAs.

The corepressor TPR1 represses MIR transcription. It was shown that nuclear SNC1 functions in defense responses through its association with TPR1, a transcriptional corepressor33. A pTPR1:TPR1-HA transgenic line with TPR1 overexpression showed constitutive activation of defense responses33. pTPR1:TPR1-HA plants were similar in size to cpr1 aba1 plants (Figs. 6a and 2a). To determine whether TPR1 overexpression also downregulates cpr1 aba1 in terms of miRNA biogenesis defects, we crossed amiR-SUL with pTPR1:TPR1-HA. The amiR-SUL pTPR1:TPR1-HA plants displayed not only a dwarf phenotype but also reduced vein-centered bleaching (Fig. 6a). We investigated the abundance of endogenous miRNAs by northern blotting. The levels of miR164, miR173, miR319, miR390, and miR159 in 15-day-old seedlings of pTPR1:TPR1-HA were reduced to 50–70% of wild type levels (Fig. 6b). In addition, real-time RT-PCR showed that the levels of seven pri-miRNAs were markedly reduced in pTPR1:TPR1-HA (Fig. 6c). These results are consistent with the model that the nuclear accumulation of SNC1 in cpr1 aba1 downregulates the abundance of miRNAs through the corepressor TPR1, which represses the transcription of MIR genes.

Reduced phasiRNA production from R genes in cpr1 aba1. Some miRNAs target protein-coding or noncoding transcripts
and trigger the production of phasiRNAs from these transcripts. Four families of noncoding TAS transcripts from eight loci generate phasiRNAs in Arabidopsis, among which TAS1A/B/C and TAS2 are targeted by miR173, TAS3A/B/C are targeted by miR390, while TAS4 is targeted by miR82814,20,34–36. Protein-coding loci also generate phasiRNAs, such as two NBS-LRR genes (At5g38850 and At5g43740) targeted by miR472 and/or miR82519,20, the PPR family targeted by miR161 and/or miR400 (Fig. 7e), and AFB2, AFB3, and CIL2 genes targeted by miR39316,19,20 (Fig. 7d). We tested the effect of cpr1 and aba1 mutations on the accumulation of three tasiRNAs, siR255 from TAS1A/B/C, siR1511 from TAS2, and 5'D8 from TAS3A/B, by northern blotting. The levels of these small RNAs were lower in cpr1 aba1 than those in Col (Fig. 7b). The levels of the miRNA triggers, such as miR173, miR390, and miR825, were also deceased in cpr1 aba1 (Fig. 7a), and this could be one factor that contributes to the reduced levels of tasiRNAs.

Next, we performed small RNA sequencing with Col and cpr1 aba1 to determine the global effects of the cpr1 aba1 mutations on phasiRNA biogenesis. The abundance of phasiRNAs generated from six PPR genes (triggered by miR161/miR400) as well as from AFB2, AFB3, and CIL2 (triggered by miR393) showed no significant difference between cpr1 aba1 and Col (Fig. 7d, e). However, the levels of tasiRNAs from four of the seven TAS loci were significantly reduced in cpr1 aba1 (Fig. 7d). The reduction was obvious throughout the phasiRNA-generating regions of the TAS transcripts (Fig. 7c and Supplementary Fig. 3b), but phasing was not affected (Supplementary Fig. 3a). The reduction in tasiRNA levels was consistent with results from northern blotting (Fig. 7b).

The levels of TAS transcripts, which are precursors to tasiRNAs, were reduced in cpr1 aba1; this may be another factor that contributes to the reduced levels of tasiRNAs in cpr1 aba1 (Fig. 7f). Besides, it is noteworthy that the levels of TAS transcripts were also reduced in pTPR1:TPR1-HA compared with Col (Supplementary Fig. 4), suggesting that overexpression of TPR1 represses the transcription of TAS genes. The accumulation of siR255 (a tasiRNA from TAS2) was reduced in pTPR1:TPR1-HA (Fig. 6b). Therefore, it is reasonable to speculate that over-accumulated and nuclear localized SNC1 in cpr1 aba1 acts with

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Fig. 6 The transcriptional corepressor TPR1 represses the transcription of MIR genes. **a** Plants of the indicated genotypes. pTPR1:TPR1-HA plants were similar in plant size to cpr1 aba1 plants (Fig. 2a). The amiR-SUL pTPR1:TPR1-HA plants displayed not only a dwarf phenotype but also reduced vein-centered bleaching. Bar = 1 cm. **b** Northern blots to detect five miRNAs and an endogenous siRNA (siR255 from TAS1A/B/C) in 15-day-old seedlings of wild type (Col) and pTPR1:TPR1-HA plants. Reduced accumulation was found for these small RNAs in pTPR1:TPR1-HA plants. **c** Relative levels of seven pri-miRNAs in pTPR1:TPR1-HA vs. wild type (Col) as determined by real-time RT-PCR. Error bars represent standard deviations calculated from three biological replicates (`Student’s t test: P < 0.05). Source data are provided as a Source Data file.
TPR1 to reduce tasiRNA biogenesis, by repressing the expression of both the miRNA triggers and the TAS loci.

In addition to At5g38850 and At5g43740, our small RNA sequencing revealed a third R gene, At1g63750, which generates phasiRNAs, with miR825* serving as the potential trigger (see Supplementary Data 3 for a complete list of phasiRNA-generating loci analyzed in this study). The levels of phasiRNAs from the three NBS-LRR genes (At5g38850, At5g43740, and At1g63750) were all decreased in cpr1 aba1 (Fig. 7c, d and Supplementary Fig. 3c).
Widespread induction of NBS-LRR genes in cpr1 aba1. To examine the effects of cpr1 and aba1 mutations on the transcriptome, either through SNC1-TPR1 or indirectly through phasiRNAs, we performed RNA-seq in wild type and cpr1 aba1 in three biological replicates. Totally, 1743 and 1020 upregulated (hyper DEGs) and downregulated (hypo DEGs) genes, respectively, were identified in cpr1 aba1 (Supplementary Data 4 and 5). The hyper DEGs were enriched in genes involved in stress responses including defense responses (Fig. 8a), consistent with previous studies on sncl–1 or cpr1,22,30,37. GO terms in various small molecule biosynthetic and metabolic processes were enriched in the hypo DEGs (Fig. 8b). RNA-seq was also performed for wild type and pTPR1:TPR1-HA, and 1719 upregulated genes (hyper DEGs) were found in the latter (Supplementary Data 6). The hyper DEGs were also enriched in GO terms related to stress responses (Supplementary Fig. 5a). Totally, 1000 genes were shared by the hyper DEGs in cpr1 aba1 and those in pTPR1:TPR1-HA, and GO analysis showed that they were also related to defense responses and other stress responses (Supplementary Fig. 5b and Supplementary Data 7).

As we observed a reduction in phasiRNAs from three NBS-LRR genes in cpr1 aba1, and as the phasiRNAs may target other genes, we interrogated the expression of all NBS-LRR genes, we identified the expression of all NBS-LRR genes. There were 165R genes with RNA-seq reads in cpr1 aba1 or Col, and we chose 156R genes with reads per million mapped reads (RPM) > 10 in each genotype (average of three replicates) for further analysis (Supplementary Data 8). The transcript levels of most R genes were increased in cpr1 aba1 relative to those in Col (Supplementary Fig. 6a). Totally, 70 and 4 upregulated and downregulated R genes in cpr1 aba1 compared with Col, respectively, were identified at fold-change > 1.5 and P < 0.05 (Fig. 8c and Supplementary Data 9). We also examined R gene expression in pTPR1:TPR1-HA and Col. Among 166R genes with RNA-seq reads in pTPR1:TPR1-HA or Col, 151R genes with RPM > 10 in each genotype (average of three replicates) were chosen for further analysis (Supplementary Data 10). Most R genes were expressed at higher levels in pTPR1:TPR1-HA relative to Col (Supplementary Fig. 6b). Totally, 68 and 2 upregulated and downregulated R genes in pTPR1:TPR1-HA relative to Col, respectively, were identified at fold-change > 1.5 and P < 0.05 (Fig. 8d and Supplementary Data 11). Thus, an overwhelming increase in R gene expression occurred in both cpr1 aba1 and pTPR1:TPR1-HA.

To determine whether phasiRNAs generated from the three source R genes can target other R genes, 47 top abundant phasiRNAs from the three loci in wild type (Col) were used for target prediction with psRNATarget38,39. The abundance cutoff was normalized RPM no less than 500; note that this abundance was comparable to, or higher than, that of tasiRNAs from TAS3B (Supplementary Fig. 3b). Totally, 95R genes (including the three source R genes) were found to be potential targets of these phasiRNAs (Supplementary Data 12). Therefore, phasiRNAs from the three source R genes can theoretically target many R genes in trans.

Among the three R genes (At1g63750, At5g38850, and At5g43740) that generate phasiRNAs, At1g63750, and At5g38850 were upregulated in both cpr1 aba1 and pTPR1:TPR1-HA as determined by RNA-seq, while At5g43740 was downregulated (Supplementary Fig. 6a, b). However, the change in At5g38850 expression was small and not independently observed by real-time RT-PCR as for the other two genes (Supplementary Fig. 7a, b). We propose that the upregulation of At1g63750 and maybe At5g38850 in cpr1 aba1 be caused by the reduced levels of miR825* (Fig. 7a), which targets these genes. The downregulation of At5g43740 was rather unexpected and may be due to repression by SNC1 or indirect effects.

The widespread induction of NBS-LRR genes in cpr1 aba1 indicated that cpr1 aba1 may exhibit enhanced resistance to pathogens. We tested the resistance of cpr1–4 and aba1–8 plants to Pst DC3000 and Pst DC3000(AvrRps4). The cpr1 aba1 double mutant was not included due to its small size, which makes it difficult to do bacterial inoculation. We observed that the aba1–8 mutant was significantly more resistant to both bacterial strains compared to wild-type plants (Supplementary Fig. 8a–d). On the contrary, the cpr1–4 mutant was similar to wild type (Supplementary Fig. 8a–d). The cpr1–2 mutant was shown to be more resistant to Pst DC3000 and Pst DC3000(AvrRpt2)25,40. Perhaps, the lack of resistance of cpr1–4 is due to its being a weaker allele. An aba1 mutant (aba1–6) was found to be more resistant to Pst DC3000(AvrRps4)28. Thus, loss (or reduction) of function in ABA1 leads to enhanced bacterial resistance.

Discussion

NBS-LRR proteins (known as R proteins) recognize pathogen infection and trigger plant defense. It has been largely unknown how R proteins affect the small noncoding portion of the transcriptome. In this study, we isolated a cpr1 aba1 double mutant in a genetic screen as defective in miRNA biogenesis. Defects in miRNA biogenesis in the double mutant are likely attributable to reduced transcription of MIR genes, as pri-miRNA levels as well as MIR167a promoter activity are reduced in the mutant. The F-box protein CPR1 negatively regulates the activation and accumulation of the R protein SNC1.25 Nuclear localization of SNC1 is required for disease resistance31 and ABA deficiency promotes the activity and nuclear localization of SNC1.28 This suggests that the phenotypes of the cpr1 aba1 double mutant could be caused by over accumulation of nuclear SNC1. Indeed, SNC1 protein over accumulated in the double mutant and loss of function in SNC1 fully rescued the double mutant’s phenotypes. Besides, nuclear accumulation of SNC1 in SNC1–NLS–GFP plants resulted in reduced levels of pri-miRNAs. Nuclear SNC1 functions in plant defense through its association with TPR1, a transcriptional corepressor33. In this study, we found that TPR1...
overexpression also led to reduced levels of miRNAs as well as pri-miRNAs. Thus, the R protein SNC1 probably represses MIR gene expression in the nucleus through TPR1.

We found that nuclear accumulation of SNC1 is also associated with reduced levels of phasiRNAs from noncoding TAS loci. This could be due to the combined effects of reduced abundance of the miRNAs that trigger phasiRNA biogenesis and repression of TAS transcription leading to decreased levels of phasiRNA precursors. In addition to noncoding TAS loci, some protein-coding genes including R genes give rise to phasiRNAs. In Legumes and Solanaceae species, phasiRNAs are produced from many R genes. In Arabidopsis, three R genes (At5g38850, At5g43740, and At1g66350) among the ~170 R genes produce phasiRNAs. The abundance of the phasiRNAs produced from the three loci was decreased in cpr1 aba1. On the other hand, a global de-repression of R gene expression was observed in the double cpr1 aba1 as determined by RNA-seq. R genes with RPM > 10 in either cpr1 aba1 or wild type (average of three replicates) were used for the identification of hyper DEGs (fold-change > 2, P value < 0.01). The hyper DEGs are enriched for genes involved in stress responses, particularly defense responses. The top 19 GO terms for downregulated genes (hypo DEGs) in cpr1 aba1. R genes with RPM > 10 in either cpr1 aba1 or wild type (average of three replicates) were used for the identification of hypo DEGs (fold-change < 0.5, P value < 0.01). GO terms in various small molecule biosynthetic and metabolic processes are enriched in the hypo DEGs. Analysis of R gene expression in three biological replicates (rep1-3) of RNA-seq of cpr1 aba1 and wild type (Col). Only DEGs with fold-change > 1.5, P value < 0.05 and RPM > 10 in either genotype (average of three replicates) were included in the analysis. Among the DEGs, 70 and 4 were upregulated and downregulated, respectively, in cpr1 aba1 relative to Col. The arrow marked an R gene (At1g63750) that could generate phasiRNAs. Analysis of R gene expression in three biological replicates (Rep1-3) of RNA-seq of pTPR1:TPR1-HA and wild type (Col). Only DEGs with fold-change > 1.5, P value < 0.05 and RPM > 10 in either genotype (average of three replicates) were included in the analysis. Among the DEGs, 68 and 2 were upregulated and down-regulated, respectively, in pTPR1:TPR1-HA relative to Col. The arrow marked an R gene (At1g63750) that could generate phasiRNAs.
is in part achieved through a miRNA-phasiRNA cascade, in which a few miRNAs target some R genes, leading to the production of phasiRNAs that perhaps target the entire R gene family. Here, we uncovered another side of the regulatory network. The activation of the R protein SNC1 leads to repression of miRNA and phasiRNA biogenesis to likely release the global repression of R gene expression to enable defense (Supplementary Fig. 9). The release of the repression itself may not be sufficient for global R gene induction, but allows for such induction to be triggered by other mechanisms.

Methods

Plant materials and growth conditions. The amiR-SUL sup-B65 mutant was isolated from an EMS mutated population of a transgenic Arabidopsis thaliana line proSUC2:amiR-SUL (amiR-SUL; a gift from Dr. Detlef Weigel) in which an artificial miRNA (amiR-SUL) is expressed from the SUCCROSE-PROTON SYMPORTER 2 (SUC2) promoter42. The cpr1 and aba1 mutations from the amiR-SUL sup-B65 line were named cpr1-1 and aba1-8, respectively. The following transgenic lines or mutants were used in this study: pMIR167a:GUS53,54, pTRI1:TPR1-HA55, SALK_045148 (cpr1-357), SALK_059469 (which we named aba1-7), snc1-21, and snc1-20. The amiR-SUL snc1-1 aba1-8 and amiR-SUL cpr1 aba1 snc1-11 genotypes were obtained by crosses. The cpr1-4 aba1-8 double mutant was generated by crossing amiR-SUL sup-B65 with Columbia (Col) wild-type plants. All seeds were grown on petri dishes containing 1/2 Murashige and Skoog Basal Medium (Sigma-Aldrich, M5519) supplemented with 50 μg/ml kanamycin. The expression levels of target transcripts were normalized to the level of the Ubiquitin 5 gene (UBQ5)56.

Identification of mutated genes in amiR-SUL sup-B65. The amiR-SUL sup-B65 mutant was backcrossed to its parental line amiR-SUL. In the F2 generation, 72 individual plants with the same phenotype as the amiR-SUL sup-B65 mutant were used for genomic DNA extraction with the CTAB method41. An equal amount of DNA from each plant was pooled for genomic DNA library construction at Beijing Genomics Institute (BGI), Shenzhen, China. The library was sequenced with the paired-ended (PE150bp) scheme on an Illumina HiSeq4000 platform at 50x coverage. The genomic region without the stop codon of SNC1 was amplified by PCR with primers PstI-NLS-BamHI-GFP-F and GFP-SacI-BamHI-GFP-F, which we designated ab1-8, were identified in amiR-SUL sup-B65. The cpr1-4 mutation abolishes a SacI restriction enzyme site, while the aba1-8 mutation generates a StuI restriction enzyme site. This formed the basis of molecular genotyping of the mutations. The primers used to amplify genomic DNA to genotype the mutations are listed in Supplementary Table 1.

DNA constructs and complementation tests. The genomic region of CPR1 was amplified using primers proCPR1-KpnI-F and CPR1-PstI-R from Col genomic DNA; the resulting fragment contains the entire CPR1 gene body without the stop codon plus 2000 bp of sequences upstream of the ATG start codon. The proCPR1: CPR1 fragment was fused with eYFP in the binary vector pGWB64042 via LR clonase (Invitrogen) to generate pCPR1:CPR1-eYFP. The genomic region of ABA1 was amplified from the Col genomic DNA with primers proABA1-KpnI-F and ABA1-SpeI-R; and the resulting fragment contains the entire ABA1 gene body without the stop codon plus 945 bp of sequence upstream of the ATG start codon. The proABA1:ABA1 fragment was fused with eYFP in the binary vector pGWB64042 via LR clonase (Invitrogen) to generate pABA1:ABA1-eYFP.

Real-time RT-PCR. Real-time RT-PCR was performed to quantify pri-miRNA and miRNA levels. One microgram total RNA was reversely transcribed with oligo (dt) using the PrimeScript 1st Strand cDNA Synthesis Kit (TAKARA, 6110A) according to the manufacturer’s instructions. Quantitative RT-PCR was performed with the 96-well StepOne Plus real-time system (Applied Biosystems) using the SYBR premix ExTaq II kit (TAKARA, RR820A). The following amplification scheme was used: 95 °C for 30 s and 40 cycles of 95 °C for 5 s and 60 °C for 30 s. The expression levels of target transcripts were normalized to the level of the internal standard UBQ5 and the 2^(-ΔΔCt) values of control samples were set to 1. All primers used in the assays are listed in Supplementary Table 3. Three independent biological replicates were carried out for each genotype or treatment, and Student’s t test was used to evaluate statistical significance.

Small RNA northern blots. Total RNAs were isolated from 15-day-old seedlings with TRI reagent (Molecular Research Center, TR118). For northern blotting, 10 μg total RNA was resolved on 15% polyacrylamide gels with 8 M urea. After gel electrophoresis, the RNAs were transferred to a Hybond-NX nylon membrane (GE healthcare). Oligonucleotide probes complementary to the miRNAs or tasiRNAs to be detected were synthesized with both 5’ and 3’ end-labeled biotin. A probe complementary to U6 was used as an internal control. Hybridization was performed for 16 h at 55 °C followed by washes. Signals were detected using the chemiluminescent Nucleic Acid Detection Module (Thermo Fisher, 89880) with a Chemiluminescence imaging system (Clinx Science Instruments Co. Ltd., China). The probes used are listed in Supplementary Table 4.

Western blots. Western blotting was performed using procedures from 15-day-old seedlings. Anti-SNC1 polyclonal antibody produced in rabbit was generated against a SNC1-specific peptide (RKTMTFIPSDFFGDC) at GenScript. Other antibodies used in immunoblotting experiments include anti-SUL45 (dilution, 1:1000) and anti-GAPDH (Santa Cruz Biotechnology, sc-65062) (dilution, 1:1000). The secondary antibodies were horseradish peroxidase-conjugated goat-anti-rabbit IgG (Bio-Rad, cat.#172-1019) (dilution, 1:2000) and goat-anti-mouse IgG (Bio-Rad, cat. #170-6516) (dilution, 1:2000). The protein signals were detected using the Amersham ECLTM Prime Western Blotting Detection Reagent (GE healthcare, RPN2232) using a Chemiluminescence imaging system (Clinx Science Instruments Co. Ltd., China).

Histochemical GUS staining. Fifteen-day-old seedlings of pMIR167a:GUS and cpr1 aba1 pMIR167a:GUS (homozygous for both the transgene and the mutant genotypes) were subjected to histochemical GUS staining according to the standard protocol46.

β-estradiol treatment. 15-day-old transgenic seedlings of SNC1–NLS-GFP and NLS-GFP grown on solid medium were transferred to 1/2 MS liquid medium containing 50 μM β-estradiol and incubated for 6 h. The treated seedlings were then subjected to fluorescence observation or RNA extraction.

Bacterial infection assays. Rosette leaves of five-week-old plants were infiltrated with bacterial suspensions of Pst DC3000 or Pst DC3000(arvR450) at OD600 = 0.001 using a needleless syringe. Inoculated plants were kept in a growth chamber with high humidity (~90%). Leaf discs from inoculated leaves were collected at day 0 and day 3 and ground in 10 mM MglCl. Serial dilutions were plated on selective plates and bacterial populations were determined as colony forming units (cfu) per cm2 leaves47.

Fluorescence microscopy. For the visualization of NLS-GFP, SNC1–GFP, and SNC1-NLS-GFP, roots of 15-day-old transgenic seedlings were observed under a Zeiss LSM 5 Pascal inverted confocal microscope.

Small RNA sequencing and data analysis. For library construction, 30 μg total RNA was resolved on a 15% polyacrylamide gel with 8 M urea. Small RNAs in the 15–40 nt size range were recovered as described48. Small RNA libraries were prepared using the NEBNext Multiplex Small RNA Library Prep Set for Illumina (New England Biolabs, E7300), and sequenced with an Illumina HiSeq2500 platform at BerryGenomics, China. Raw reads (SE50) were trimmed using Perl scripts to remove adapters. The clean reads were mapped to the Arabidopsis thaliana genome TAIR10 using the Bowtie program49. The small RNA reads were calculated and normalized in reads per million mapped reads (RPM). The comparison between sets was conducted with the R package edgeR50.

For analysis of miRNA levels, small RNA reads were mapped to annotated miRNAs in miRBase51. For analysis of tasiRNAs levels, small RNA reads were mapped to each of the eight annotated TAS genes (TASA1–B/C, TAZ2, TASA1/3B/C, and TAS4). For the analysis of phasiRNAs from protein coding genes, such as R genes and PPR genes, small RNA reads were mapped to the genes known to produce phasiRNAs and only uniquely mapped reads were included in subsequent analyses. Phasing analysis was performed as described51. Small RNA reads from sense and antisense strands were unified. Phasing score was calculated by the following formula:

\[
\text{Phasing score} = \ln \left( 1 + 10^\left( \frac{\text{num. } P_i}{\text{num. } O_i} \times \sigma^{-2} \right) \right), n=3,
\]

where num. is number of phase cycle positions occupied by no less than one read
within a 10-cycle window, \( P \) = total number of all small RNA reads with start coordinates in a given phase within a 10-cycle window; \( U \) = total number of small RNA reads with start coordinates out of each phase. In our analysis, phase cycle was set at 21 nt.

For predicting \( R \) genes that can be potentially targeted by phased siRNAs generated from the three source \( R \) genes, \( 47 \) top abundant phased siRNAs (normalized reads with \( P \) no less than 500) from small RNA sequencing of wild type (Col) were used for target prediction with the online server psRNATarget (http://plantgrn.noble.org/psRNATarget)\(^{38,39}\). The score for complementarity between phased siRNA and target mRNA sequences was set to be no more than 4.

**RNA-seq data analysis.** Total RNAs extracted from 15-day-old seedlings of wild type and \( R \) mutants were sent to Novogene Tech Co. Ltd. and sequenced on Illumina Hiseq \( x \)-10 platform to generate single-end reads of 150 bp in length. The analysis was performed by a homemade pipeline PHiSDA (https://github.com/grubbio/pHISDAbldb/master/phsda). Clean reads were trimmed to remove adapter sequences and mapped to Arabidopsis genome TAIR 10 using HISAT2 with default settings\(^ {52}\). Transcript levels were calculated and normalized in RPM. Then gene expression levels were normalized and differentially expressed genes (DEGs) were calculated with the cut-off of fold-change > 1.5 (for hyper DEGs) or fold-change < 0.67 (for hypo-DEGs), and \( P < 0.05 \) using DESeq2\(^ {53}\). For GO-term analysis, only genes with fold-change > 2.0 (for hyper DEGs) or fold-change < 0.5 (for hypo-DEGs), \( P < 0.01 \), and RPM > 10 in either genotype (average of three replicates for each genotype) were included. For heatmap figures, \( R \) genes with fold-change > 1.5 (for hyper DEGs) or fold-change < 0.67 (for hypo-DEGs), \( P < 0.05 \), and RPM > 10 in either genotype (average of three replicates for each genotype) were included. The GO-term enrichment analysis was performed online at TAIR (http://www.arabidopsis.org/tools/go_term_enrichment.jsp). Only the top 20 terms were presented in this paper.

**Data availability**

All raw and processed data were deposited in NCBI GEO with the accession number GSE111240 [https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc = GSE111240]. All other data supporting the findings of this study are available within the manuscript and its supplementary files or are available from the corresponding authors upon request. A reporting summary for this Article is available as a Supplementary Information file. The source data underlying Figs. 1d, 2b–d, 3a, b, d, 4c–f, 5b, 6b, c, 7a, b and f, and Supplementary Figs. 4, 7a, 7b, 8b, and 8d are provided as a Source Data file (Supplementary Data 13).

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Author contributions
Q.C., C.L., and X.C. designed the project and experiments. Q.C., S.W., Y.H., L.L., and W. H. performed the experiments. C.L. and L.G. analyzed the data. Q.C., C.L., W.M., R.M., and X.C. wrote the manuscript.

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