The TIR-NB-LRR Gene *SNC1* Is Regulated at the Transcript Level by Multiple Factors

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SNC1 (*suppressor of NPRI, constitutive 1*) is a haplotype-specific Toll and interleukin-1 receptor-like nucleotide-binding site leucine-rich repeat type of resistance (*R*)-like gene possibly mediating race-specific disease resistance. Inactivation of its negative regulator *BON1* (*BONZAI1*)/*CPNI* and *BAP1* genes or upregulation of its expression epigenetically lead to constitutive defense responses and dwarf phenotype. Here, we report an autoactivation of *SNC1* by introducing it into *Arabidopsis* as a transgene. The *SNC1* genomic fragment confers a dwarf phenotype induced by defense response upregulation associated with a higher *SNC1* transcript level. Analysis of the β -glucuronidase reporter gene under the control of the *SNC1* promoter suggests three modes of regulation on the *SNC1* transcript level: a repression by the chromosomal structure, a feedback amplification from *SNC1* on its promoter sequences, and a repression by *BON1*. These regulations appear to be independent of each other. The regulation of *SNC1* possibly exemplifies a universally complex control of *R* genes to ensure a repression of *R* activation under nonstress conditions and a robust activation of defense responses once the *R* gene is induced.

Race-specific pathogen resistance is mediated by an interaction between a plant disease resistance (*R*) gene and its corresponding pathogen avirulence (*Avr*) gene (Flor 1971). The gene-for-gene interaction often leads to defense responses, including hypersensitive response (HR), to restrict the growth of the pathogen (Hammond-Kosack and Jones 1996). A number of *R* genes have been molecularly cloned in many species. In spite of the diverse array of pathogens they perceive, the *R* genes mostly encode five classes of proteins, with *R* proteins in the largest family containing nucleotide-binding site (NB) and leucine-rich repeat (LRR) domains (Dangl and Jones 2001; Martin et al. 2003). The amino-termini of these proteins are of either the Toll and interleukin-1 receptor-like (TIR) type or the coiled-coiled (CC) type. There are 149 NB-LRR-encoding genes in the Columbia (Col) accession of *Arabidopsis thaliana* (Meyers et al. 2003). The biological functions of most of these genes have not been identified, but it generally is thought that they are used to detect a vast variety of pathogens. Recently, they also have been implicated in downstream signaling pathways in defense response (Peart et al. 2005).

R gene activities are highly regulated for optimal plant defense and plant growth. Recognition of cognate *Avr* proteins activates *R* proteins to induce downstream defense responses. The interaction between *R* and *Avr* could be direct, similar to a ligand-receptor interaction (Deslandes et al. 2003; Dodds et al. 2006; Jia et al. 2000). It also could be indirect, where an *R* protein is guarded by a plant guard protein whose activity can be modulated by pathogen effector proteins (Belkadir et al. 2004; Dangl and Jones 2001; Martin et al. 2003; Schneider 2002; van der Biezen and Jones 1998). The activity of *R* proteins also can be regulated at the intramolecular level. Structure-function analysis indicates that the LRRs control the molecular state of the NB domain and NB might serve as a molecular switch which undergoes conformational changes upon pathogen recognition (Takken et al. 2006). Recent studies reveal another aspect of *R* protein regulation that involves subcellular compartmentation and protein-protein associations (Burch-Smith et al. 2007; Shen et al. 2007).

The level of *R* gene transcripts is usually low under nonpathogen invasion conditions, presumably preventing growth inhibition caused by high expression of *R* genes. Overexpression of *R* genes has been shown to lead to induction of plant defense responses in the absence of pathogen invasion, and the resistance tends to be broad spectrum. This effect has been observed for *Prf* (Oldroyd and Staskawicz 1998), *Pto* (Tang et al. 1999), and *Cf-9* (Wulff et al. 2004). Constitutive activation of *R* gene activity and defense response often are accompanied by dwarfism and sometimes lethality, a fitness cost underlying the necessity for tight regulation of *R* genes (Heil and Baldwin 2002). *R* genes can be modulated at the transcript level. Salicylic acid can induce the expression of a number of *R* genes including *SSI4*, *RPW8*, and *SNC1* (*suppressor of NPRI, constitutive 1*), possibly acting as an amplification of defense response (Shirano et al. 2002; Xiao et al. 2003; Yang and Hua 2004). The transcript level of *R* gene also has been found to be regulated by pathogen. A rice *R* gene, *Xa27*, encoding a small protein without significant motifs is shown to be induced by its cognate *AvrXa27* effector protein, and its inducibility appears to determine the resistance or susceptibility of the rice cultivar (Gu et al. 2005).

SNC1 (*BAL*) is a TIR-NB-LRR type of *R* gene residing in the *RPP5* gene cluster that is very divergent among accessions in *Arabidopsis* (Noel et al. 1999; Stokes et al., 2002; Zhang et al. 2003). The *RPP4* and *RPP5* genes are the first genes of this cluster from the Col and the Landsberg *erecta* (*Ler*) accessions, respectively, and they are responsible for resistance to different variants of the downy mildew (Parker et al. 1997; van der Biezen et al. 2002). *SNC1* is the second gene in the Col cluster and no pathogen specificity has been assigned to this gene. However missense mutation in the *snc1-1* mutant induces constitutive defense responses likely

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due to an activation of the *SNCI* protein (Li et al. 2001; Zhang et al. 2003). An epigenetic allele of *SNCI*, namely *bal* or *sncl-2*, exhibits a higher expression of *SNCI* than the wild type and also leads to constitutive defense responses and a dwarf phenotype (Stokes et al. 2002). These observations suggest that *SNCI* is a functional *R* gene that can induce defense responses when activated.

Intriguingly, *SNCI* is under a negative control of the *Arabidopsis* *BON1/CPN1* gene and its functional partner *BAP1* gene (Yang and Hua 2004; Yang et al. 2006). *BON1* is an evolutionarily conserved copine gene and the loss-of-function *bon1* mutants have enhanced disease resistance and a dwarf phenotype in a temperature- and humidity-dependent manner (Hua et al. 2001; Jambunathan et al. 2001). *BAP1* encodes a small lipid-binding protein and its loss-of-function mutants have a phenotype similar to but weaker than the *bon1* mutants (Yang et al. 2006). In the *bon1* and *bap1* loss-of-function mutants, the *SNCI* gene presumably is activated, leading to constitutive defense responses and reduced plant growth (Yang and Hua 2004; Yang et al. 2006). *SNCI* transcript is higher in *bon1-1* and *bap1-1* than in the wild type (Yang and Hua 2004; Yang et al. 2006). However, it is not known whether the repression of *SNCI* by *BON1* and *BAP1* is primarily at the level of the *SNCI* transcript or the *SNCI* protein activity. *SNCI* is shown to be regulated at the transcript level by temperature and salicylic acid (SA). There is a lower expression of *SNCI* at 28 than at 22°C and SA application induces the level of its transcript (Yang and Hua 2004). The *SNCI* transcript level appears to be regulated epigenetically as well. The mutant phenotype of *bal/sncl-2* isolated from the *ddm1* background was due to an overexpression of *SNCI*, but there was no nucleotide change identified in the *SNCI* gene (Stokes et al. 2002). Ethyl methane sulfonate (EMS) mutagenesis of *bal* induced a high incidence of reversion, further indicating that *SNCI* is under an epigenetic control (Stokes et al. 2002).

In this study, we further investigated the regulation of the *R* gene *SNCI*. We found that *SNCI* as a transgene has a much higher expression than the endogenous gene, and this confers a dwarf phenotype as a consequence of upregulation of defense responses. Using transgenic plants with the *SNCI* promoter-reporter gene fusion, we further demonstrated a repression of *SNCI* transcript level at the endogenous location and a regulation of *SNCI* by *BON1* and *SNCI* through the promoter of *SNCI*.

Fig. 1. *pSNCI::SNCI* confers a dwarf phenotype. **A**, Growth phenotypes of *pSNCI::SNCI* transgenic plants in *bon1-2* and the wild-type *Ws* at 22°C. Shown are representative transgenic lines next to the nontransgenic line. **B**, Growth phenotypes of *pSNCI::SNCI* transgenic plants in the *Col-0* background. At 22°C, hemizygous transgenic plants had a *bon1-1*-like phenotype and homozygous transgenics had a more severe phenotype. At 28°C, they both showed wild-type phenotypes.

RESULTS

Transgenic plants

with *pSNCI::SNCI* exhibit dwarf phenotype.

SNCI is shown to mediate the dwarf phenotype of the *bon1* mutant in the *Arabidopsis* accession *Col-0*, and the *bon1-2* mutant in the *Ws* accession does not exhibit an obvious growth defect due to a lack of functional *SNCI* (Yang and Hua 2004). We hypothesize that introducing the wild-type *SNCI* gene to *bon1-2* should recapitulate the *bon1-1* dwarf phenotype. To test this, we isolated the genomic fragment of the *SNCI* gene starting from 1,912 bp 5' to the ATG translation start site and ending at 1,005 bp 3' to the stop codon and transformed this *pSNCI::SNCI* fragment into the wild-type *Ws* and *bon1-2*. As anticipated, most of the T1 plants (16 of 25) of *pSNCI::SNCI* in *bon1-2* exhibited a *bon1-1*-like phenotype, including dwarfed stature and curled leaves with water-soaked appearance (Fig. 1A). Surprisingly, introduction of *pSNCI::SNCI* into the wild-type *Ws* plants caused a mutant phenotype as well. In all, 10 of 25 independent T1 plants exhibited a *bon1-1*-like phenotype (Fig. 1A). We subsequently introduced *pSNCI::SNCI* into the wild-type *Col* background. Of the 17 T1 plants, 15 exhibited dwarf phenotype to various degrees (Fig. 1B). Thus, *pSNCI::SNCI* is capable of conferring a dwarf phenotype to wild-type plants independent of the repression function of *BON1*.

The progenies of *pSNCI::SNCI* primary transformants in *Col* gave rise to three types of phenotype: wild type, *bon1-1*-like, and extremely dwarf (Fig. 1B). Wild-type-looking plants were not transgenic, confirmed by sensitivity of their progenies to antibiotics used for transgene selection. The *bon1-1*-like plants were similar in phenotype to their parents—small in stature, with curly and water-soaked leaves—but were fertile. They gave rise to progenies with all three types of phenotype and, thus, likely are hemizygous for the transgene. The extreme dwarf plants were arrested in growth at an early seedling stage and they died before bolting when grown at 22°C but grew like the wild type at 28°C (Fig. 1B). They gave rise to progenies with a uniform extreme dwarf phenotype, indicating that they are homozygous for the transgene. Thus, the severity of the phenotype is correlated with the zygosity or copy number of the transgene.

pSNCI::SNCI transgenic plants have enhanced disease resistance.

We reasoned that the dwarf phenotype of *pSNCI::SNCI* transgenics likely results from a constitutive defense response because it is very similar to that of *bon1-1*, *sncl-1*, and *sncl-2* (Stokes et al. 2002; Yang and Hua 2004). To test this hypothesis, we compared the growth of virulent *Pseudomonas syringae* pv. *tomato* DC3000 in the transgenics with that of the wild-type *Col*. At 3 days after dip inoculation, *P. syringae* pv. *tomato* DC3000 grew to 10^8 while five independent *pSNCI::SNCI* lines grew to 10^4 to 10^5 respectively (Fig. 2A). The transgenics were as resistant as *bon1-1* that had 10^5 growth of the pathogen (Fig. 2A). Consistent with the disease resistance phenotype, we found that the *pSNCI::SNCI* transgenics had a higher expression of the *pathogenesis-related 1* (*PR1*) gene, a molecular marker for defense response (Uknes et al. 1992) (Fig. 2B).

To further test the hypothesis that the dwarf phenotype results from constitutive defense responses, we crossed a hemizygous *pSNCI::SNCI/Col* transgenic into a *Col* plant containing a bacterial *NahG* gene encoding an SA-degrading salicylate hydroxylase (Lawton et al. 1995). F1 plants with *pSNCI::SNCI* (hemizygous) containing *NahG* exhibited a much weaker phenotype than hemizygous transgenics without *nahG* (data not

shown). Consistently, the upregulation of *PR1* expression in *pSNC1::SNC1* transgenics was suppressed by *NahG* (Fig. 2B). Thus, the dwarf phenotype likely results from activated defense responses mediated by SA.

pSNC1::SNC1 transgenic plants have a high expression of *SNC1*.

We analyzed the transcript level of *SNC1* in *pSNC1::SNC1* transgenic lines by RNA blot hybridization and found that it was elevated in plants with dwarf phenotypes (Fig. 3A). The *SNC1* transcript level is low in the wild-type Col plants but is elevated in *bon1-1*. Hemizygous transgenic plants, with a *bon1-1* like phenotype, had an *SNC1* transcript level similar to that of *bon1-1*. Type III plants, with a more severe phenotype, had the highest *SNC1* transcript level. Thus, the degree of the morphological defect is correlated with the level of the *SNC1* RNA transcript. More *SNC1* transcript is accumulated in the homozygous plants with a more severe phenotype than in the hemizygous plants.

Fig. 2. *pSNC1::SNC1* transgenic plants have enhanced resistance to a virulent pathogen. **A**, Growth of *Pseudomonas syringae* pv. tomato DC3000 in Col-0, *bon1-1*, *pad4-1*, and five independent *pSNC1::SNC1* transgenic lines. *pad4* is more susceptible to the pathogen than Col, while all five lines and *bon1* are more resistant than Col. The data shown is from one of two biological repeats. **B**, *pSNC1::SNC1* transgenic plants have upregulated *PR1* expression. RNA blot analysis shows that *PR1* is upregulated in the *pSNC1::SNC1* transgenic compared with the wild type, but this upregulation is abolished with *NahG* that degrades salicylic acid. *rRNAs* are used as controls for equal loading (same in subsequent RNA blot analyses).

The fact that *pSNC1::SNC1/Col* has a very high *SNC1* transcript level prompted us to determine whether these lines contain high copies of transgenes, as was reported for the *RPW8* transgenics (Xiao et al. 2003). We carried out a Southern blot analysis on three *pSNC1::SNC1/Col* transgenic lines. A *SNC1*-specific probe revealed two fragments, one representing the endogenous *SNC1* at 3.8 kb and the other representing the transgene at 2.2 kb. Using the program ImageJ, we quantified *SNC1* signals on the blot. The signal ratios of transgene versus the endogenous gene were 2.1, 1.4, and 1.9 for line no. 1, no. 2, and no. 11, respectively (Fig. 3B). Because these three lines likely only contain one or two *pSNC1::SNC1* transgenes, the dwarf phenotype does not require very high copy numbers of the *SNC1* gene. Furthermore, the mutant phenotype is unlikely due to having three copies of *SNC1* (two endogenous copies and one transgene copy) in transgenics rather than two copies in Col because *pSNC1::SNC1* conferred a dwarf phenotype in the Ws background. Some T1 transgenics in Ws or *bon1-2* would have only one copy of *SNC1* because there is no endogenous *SNC1*, and yet they still exhibited dwarf phenotypes.

pSNC1::SNC1:GFP transgenic plants reveal a feedback regulation on both the transgene and the endogenous gene.

We hypothesize that the high expression of the *pSNC1::SNC1* transgene is partly due to a feedback regulation from activated defense responses. To test this, we constructed a chimeric gene *pSNC1::SNC1:GFP* similar to *pSNC1::SNC1*, except that a green fluorescent protein (GFP) is fused in frame to the carboxyl terminus of *SNC1*. Transgenics of *pSNC1::SNC1:GFP* were generated in the Col background and some of them exhibited a dwarf phenotype at 22°C, similar to the *pSNC1::SNC1* transgenics, and this dwarf phenotype could be suppressed by a higher growth temperature of 28°C (Fig. 4A). This suggests that *pSNC1::SNC1:GFP* has an activity similar to *pSNC1::SNC1* and it is possible to distinguish the endogenous *SNC1* from the *SNC1* transgene in *pSNC1::SNC1:GFP* transgenics.

We analyzed the expression of the endogenous *SNC1* and the *pSNC1::SNC1:GFP* transgene in transgenic plants. We found they both had a higher expression in transgenics than the endogenous gene in the wild type at 22°C (Fig. 4B). Thus, the presence of the transgene increases the expression of the endogenous *SNC1* gene. However, the endogenous *SNC1* was expressed at a similar level in the transgenic plants and the wild type at 28°C (Fig. 4B). At this higher temperature, the transgenic plants were wild-type looking, presumably because of a suppression of defense responses. Thus, the upregulation of the endogenous and the transgene

Fig. 3. *pSNC1::SNC1* transgenic plants have a high *SNC1* expression level. **A**, *SNC1* transcript levels in the wild-type Col, *bon1-1*, and transgenic lines no. 2 and no. 11 in homozygous (ho) and hemizygous (he) status. **B**, Southern blot analysis of the genomic DNAs of three *pSNC1::SNC1* transgenic lines. The 3.8-kb fragment represents the endogenous *SNC1* gene and the 2.2-kb fragment represents the transgene. Signal ratios of the two genes are indicated.

SNC1 at 22°C likely is due to a feedback from the activation of defense responses. This is further supported by higher transcript levels of *SNC1::GFP* and *SNC1* at 22°C compared with 28°C (Fig. 4B).

pSNC1::GUS transgene has a higher expression than the endogenous *SNC1*.

We noted that the *SNC1::GFP* transgene had a higher expression than the endogenous *SNC1* at 28°C, suggesting that a repression of expression at the endogenous *SNC1* location is released in the transgene location. We hypothesize that an initial increase of the *SNC1* transcript level in transgenic plants is amplified through feedback regulation from defense response activation, leading to further increase of the *SNC1* transcript level.

To further test this hypothesis, we made a fusion construct driving the β -glucuronidase (GUS) reporter gene by the promoter and the first exon (encoding approximately $1/10$ of the protein) of *SNC1*. This *pSNC1::GUS* was introduced into Col and more than 18 transgenic lines were generated, and they all showed wild-type phenotypes. The *GUS* gene was found to be ubiquitously expressed in 15 of 18 lines, and young leaves and stems appeared to have slightly higher activities than older tissues (Fig. 5A). No GUS activity was observed in flowers except for the sepals.

Two representative lines with a single T-DNA insertion (determined by transgene segregation) were chosen for further analysis. We compared the transcript level of the *SNC1* endogenous gene and the *pSNC1::GUS* transgene by RNA blot analysis with a probe complementary to the first exon of *SNC1*. Consistent with the hypothesis, the *pSNC1::GUS* transgene had a much higher expression level than the endogenous *SNC1* gene (data of one line is shown in Figure 5B). These data, in conjunction with a higher expression of *pSNC1::SNC1::GFP* compared with *SNC1* at 28°C (Fig. 4B), suggest that there is a repression of the *SNC1* gene at the transcriptional level in its endogenous location.

Fig. 4. *SNC1* is upregulated through a feedback regulation. **A**, *pSNC1::SNC1::GFP* transgenic plants have a dwarf phenotype at 22°C but not 28°C. On the left is a wild-type Col plant and on the right are the two representative transgenic lines (m1 and m2). **B**, *pSNC1::SNC1::GFP* transgenic plants have a higher *SNC1* and *SNC1::GFP* expression at 22 than at 28°C. Notice that the endogenous *SNC1* expression at 22°C is higher in the transgenic than in the wild type.

Regulation of the *pSNC1::GUS* transgene by *BON1*.

SNC1 is negatively regulated by *BON1*, possibly at the transcript level or protein activity level. To determine whether the *SNC1* transcription can be repressed by *BON1*, we compared the *pSNC1::GUS* expression in *bon1-1* and the wild-type Col. More than 10 independent transgenic lines of *pSNC1::GUS* were generated in the *bon1-1* background and there appeared to be a higher expression of GUS in *bon1-1* than in Col in general. However, the GUS activities exhibited variability among different lines likely due to positional effects of the transgene. To unambiguously compare the level of *pSNC1::GUS* in the wild-type Col and *bon1-1*, we generated near isogenic lines (NILs) with the same transgene in Col and *bon1-1* backgrounds for three independent lines. This was achieved by crossing *pSNC1::GUS/Col* to *bon1-1* and isolating transgenics in *BON1* and *bon1-1*, respectively, through polymerase chain reaction (PCR) genotyping.

GUS staining indicates that there was a moderate upregulation of GUS activity in *bon1-1* compared with the wild-type Col (Fig. 6A). We confirmed this by RNA blot analysis on plants approximately 3 weeks old. The *pSNC1::GUS* transgene had a higher transcript level in *bon1-1* than in Col, similar to the endogenous *SNC1* (Fig. 6B). Thus, the *BON1* gene represses the transgene *pSNC1::GUS* in addition to the endogenous *SNC1* gene, and the promoter region of *SNC1* appears to contain elements responsible for this repression.

We further asked whether the higher expression of *pSNC1::GUS* in *bon1-1* is through a feedback from *SNC1*, as suggested by a previous study on the endogenous *SNC1* gene (Yang and Hua 2004). We introduced a loss-of-function *snc1-11* mutation into transgenic lines and generated NILs of *pSNC1::GUS* in the wild-type Col, *snc1-11*, *bon1-1*, and *bon1-1snc1-11*. Southern blot analysis confirmed that all NILs of the four genotypes from the same T1 plant indeed contained the same transgene (data not shown). Presumably, feedback regulation from activation of defense responses is blocked by *snc1-11*, as indicated by the wild-type phenotype of *bon1-1snc1-11*. Consistent with GUS staining in these NILs (Fig. 6A), RNA blot analysis on 3-week-old plants revealed different levels of *pSNC1::GUS* transcript in different genetic backgrounds (Fig. 6B). Gene *bon1-1* had the highest expression of *pSNC1::GUS*; Col and *bon1-1snc1-11* had comparable levels lower than that of *bon1-1*; and *snc1-11* had the lowest expression. *pSNC1::GUS* has a higher expression

Fig. 5. *pSNC1::GUS* expression in transgenic plants. **A**, Expression of *pSNC1::GUS* at different developmental stages of a representative line assayed by β -glucuronidase (GUS) staining. **B**, *pSNC1::GUS* has a higher expression than the endogenous *SNC1* in a representative line. A probe is used to detect both *SNC1* and *SNC1::GUS* transcripts.

in Col than in *snc1-11*, indicating that *SNC1* can feedback to regulate itself through its promoter. *pSNC1::GUS* has a higher expression in *bon1-1* even in the absence of a functional *SNC1*, indicating that *BON1* can regulate the promoter of *SNC1* without a feedback regulation.

Interaction of different regulations on *SNC1* gene activity.

SNC1 activity is regulated by multiple modes at its endogenous locus: a transcriptional repression at the *SNC1* locus (indicated by the *pSNC1::SNC1* and *pSNC1::GUS* transgenic lines), epigenetic repression (as in *bal* and *cpr1*), and protein activity (as in *snc1-1*). To determine whether *BON1* regulates *SNC1* through one of these modes, we analyzed the interaction of the regulations by *BON1* and by the *SNC1* locus.

We first compared the phenotypes of *pSNC1::SNC1* transgenic plants in *bon1-2* and the wild-type Ws. For most of the lines, phenotypes were more severe in *bon1-2* than in Ws, especially in inflorescence (Fig. 7A). Flowers of the *pSNC1::SNC1/bon1-2* transgenics were smaller than those of the *pSNC1::SNC1/Ws* transgenics, and they tended to open their sepals and petals at an earlier developmental stage than the wild type. These phenotypes frequently were observed in enhancers of *bon1* (unpublished data), indicating a stronger phenotype of *pSNC1::SNC1/bon1-2*.

Gene *snc1-1* has a missense mutation which might cause an activation of the *SNC1* protein activity (Zhang et al. 2003). We generated a *snc1-1bon1-1* double mutant, and found it had a more severe phenotype than either of the single mutants (Fig. 7B). At 22°C, the double mutant was even smaller than the single mutants and grew like a tiny ball. At 28°C, *snc1-1* and *bon1-1* grew like the wild type but the double mutant showed a dwarf phenotype.

Gene *bal* (*snc1-2*) is an epi-allele of *SNC1* with a high expression of the *SNC1* transcript (Stokes et al. 2002); whereas

cpr1-1 was shown to be a possible *snc1* epi-allele (Stokes and Richards 2002) with a high expression of *SNC1* (Yang and Hua 2004). Because there is no nucleotide sequence alteration of *SNC1* in *snc1-2* or *cpr1-1*, we could not identify the *bon1-1snc1-2* or *bon1-1cpr1-1* double mutants via genotyping F2 plants from crosses between *bon1-1* and *snc1-2* or between *bon1-1* and *cpr1-1*. However, we observed plants smaller than the singles in both populations at 22°C at a frequency of approximately $1/16$ and dwarf plants also were observed in F2 plants grown at 28°C (data not shown). Thus, it is likely that both the *bon1-1snc1-2* and the *bon1-1cpr1-1* doubles have more severe phenotypes than their respective single mutants, and further experiments will confirm the identities of the potential double mutants.

DISCUSSION

Here, we report the investigation the regulation of a TIR-NB-LRR type of *R* gene, *SNC1*, at the transcript level and the mode of regulation of *SNC1* by its negative regulator, *BON1*. *SNC1* is a haplotype-specific gene found in the Col-0 accession of *A. thaliana*. When it is integrated into an exogenous chromosomal location as a transgene, it induces a dwarf phenotype likely due to an upregulation of defense response, indicating an auto-activation of the *SNC1* gene. With the use of the *GUS* reporter gene, our study indicates three levels of transcriptional regulation on *SNC1*: a repression by the chromosomal structure, a feed-back amplification from *SNC1*, and a repression by *BON1*.

SNC1 is under a negative control at its endogenous location. This is demonstrated by the higher expression of *pSNC1::GUS* compared with the endogenous *SNC1* in transgenic plants and

Fig. 6. *SNC1* is transcriptionally regulated by *SNC1* and *BON1*. **A**, Expression of *pSNC1::GUS* in near isogenic lines (NILs) in Col, *bon1-1*, *snc1-11*, and *bon1-1snc1-11* background for a representative line assayed by β -glucuronidase (*GUS*) staining. **B**, Expression of *pSNC1::GUS* and the endogenous *SNC1* in NILs in Col, *bon1-1*, *snc1-11*, and *bon1-1snc1-11* backgrounds for a representative line assayed by RNA blot analysis. A *SNC1*-specific probe hybridizes to the endogenous gene *SNC1*, the transgene, and the smaller *SNC1* transcript in *snc1-11*.

Fig. 7. Interaction among different regulations on *SNC1*. **A**, Inflorescences of *pSNC1::SNC1/Ws* and *pSNC1::SNC1/bon1-2*. Images in the black background (left) and those in the blue background (right) are of the same magnification. **B**, Interaction of *snc1-1* and *bon1-1*; *bon1-1snc1-1* has a more severe phenotype than the single mutants at both 22 and 28°C. Note that, at 28°C, the single mutants are wild-type looking whereas the double mutants exhibit a dwarf phenotype.

the wild type. Consistently, *pSNCl::SNCl:GFP* as a transgene has a higher expression than the endogenous *SNCl*, even at 28°C. We propose that the expression of *SNCl* is under a negative control at its endogenous location, possibly through chromatin structure or DNA methylation modification. The discovery of epi-alleles of *SNCl* supports the hypothesis that *SNCl* expression is repressed by its chromatin structure or a high methylation status (Stokes et al. 2002). *SNCl* is located in a complex locus containing repeats of TIR-NB-LRR genes with homology to each other (Noel et al. 1999). In addition, two retrotransposon elements are embedded in this gene cluster. Both gene repeats and transposable elements could be the source of epigenetic control. The gene *bal*, an epi-allele with high *SNCl* expression, was identified from a *ddm1* background, suggesting that demethylation may have induced an open chromatin structure for the transcription of *SNCl*. Two other potential *SNCl* epi-alleles are *ssi1* and *crp1*, and their existence further supports the hypothesis that this gene is subject to epigenetic controls (Stokes and Richards 2002). It is very likely that when *SNCl* is integrated into another chromosomal location, as in *pSNCl::SNCl* transgenic plants, repression normally imposed by its endogenous chromatin structure is lost and the exogenous location could assume an open chromatin structure for transcription.

SNCl is under a feedback amplification through its promoter sequences. This was suggested in the *bon1-1* study, where an upregulation of the *SNCl* transcript is dependent on the SA-mediated defense responses (Yang and Hua 2004). This study shows that one or two copies of the *SNCl* transgene (even in the absence of the endogenous *SNCl* gene, as in the Ws accession) can lead to upregulation of defense responses and a high *SNCl* expression. This is in contrast to previous studies, where several *R* genes were shown to induce constitutive defense response. In those cases, *R* genes either were expressed under an exogenous strong promoter such as the CaMV35S promoter, or required multiple copies of the transgene with its endogenous promoter (Oldroyd and Staskawicz 1998; Tang et al. 1999; Wulff et al. 2004; Xiao et al. 2003). Furthermore, *SNCl* expression in *pSNCl::SNCl* transgenic is higher under conditions inducing a dwarf phenotype than conditions conferring a wild-type phenotype, suggesting a feedback from the downstream defense. We generated transgenic lines of three other TIR-NB-LRR types of *R* genes in the same *RPP5* cluster under their native promoters and found that none of them induces dwarfism (*unpublished data*). The specific property of the *SNCl* transgene supports an *SNCl*-specific autoactivation activity through its promoter. Alternatively, *SNCl* may have a specific activity tied to growth control in addition to defense response control.

Using *pSNCl::GUS* transgenics, we show that the feedback could be mediated by the *SNCl* promoter. *pSNCl::GUS* has a higher expression in the wild-type *SNCl* background than in *snc1-11*, indicating that *SNCl* can positively regulate its own promoter. The overexpression of *SNCl* in *pSNCl::SNCl* transgenics likely is due to a feedback amplification through the promoter sequences. This feedback amplification appears to be essential for the autoactivation of *SNCl* because overexpression by the CaMV 35S promoter is less potent than the *SNCl* promoter in inducing autoactivation. A smaller proportion of *p35S::SNCl* transgenic lines segregated dwarf phenotypes at a lower frequency when compared with *pSNCl::SNCl* transgenics (*unpublished results*). This suggests that it requires many copies of the transgene to drive extremely high expression of *SNCl* to induce activation in *p35S::SNCl* transgenics. Similar feedback amplification of *R* genes has been reported for *SSI4* and *RPW8*, and they are essential to generate the hypersensitive cell death phenotype induced by the *RPW8* transgene

(Shirano et al. 2002; Xiao et al. 2003). Autoactivation of *SNCl* may involve a similar mechanism. An increase in expression of the *pSNCl::SNCl* transgene could slightly upregulate defense response which further enhances the expression of *SNCl* through the *SNCl* promoter.

SNCl appears to be repressed at the transcriptional level by the *BON1* gene. *pSNCl::GUS* expression is higher in *bon1-1* than in wild-type *BON1*. Using *snc1-11* to block the feedback regulation, *pSNCl::GUS* still has a higher expression in *bon1-1snc1-11* than in *snc1-11*. Thus, it appears that *BON1* can negatively regulate *SNCl* at the transcriptional level. This regulation is likely to be indirect because *BON1* is mostly localized to the plasma membrane and its associated protein BAP1 is mostly membrane bound. Transcriptional control by *BON1* could be relayed by molecules generated with the perturbation of homeostasis with the loss of *BON1* function. However, caution has to be taken to interpret these data because *pSNCl::GUS* may not mimic endogenous *SNCl* at the transcriptional control due to the release of local repression of the transgene. It also is yet to be determined whether this transcriptional regulation is the primary and sole mode of *BON1* regulation of *SNCl*.

This study further suggests that different transcriptional controls might work in parallel. Although it has yet to be confirmed by comparing NILs, *pSNCl::SNCl* in general has a much more severe defect in *bon1-2* than in the wild-type Ws, especially in flower development. Derepression of *pSNCl::SNCl* from its endogenous chromosomal region in Ws likely is further enhanced by an upregulation of the *SNCl* activity from the *bon1-2* mutation, indicating that these two regulations are distinct or at least not totally overlapping. Gene *bon1-1* has a synergistic effect with *snc1-1*, suggesting that the mode of *BON1* repression of *SNCl* is different from the protein activation or derepression by the *snc1-1* missense mutation. Also, *bon1-1* appears to have a synergistic effect on *snc1-2/bal*, suggesting that *BON1* regulation and epigenetic control of *SNCl* are likely different.

In sum, the regulation of *SNCl* possibly exemplifies a universal complex control of *R* genes. In addition to the recent discoveries of *R* activity regulation at the protein level, such as posttranslational modification, subcellular localization, and protein–protein interactions, *R* activity also can be regulated at the RNA transcript level, as is found for *SNCl*. These sophisticated regulations likely ensure a prevention of fitness cost by *R* activation under nonstress conditions and a robust activation of defense response once the *R* gene is induced.

MATERIALS AND METHODS

Plant growth conditions.

A. thaliana plants were grown at 22 or 28°C under constant light or a photoperiod of 12 h of light at 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$, with 50 to 70% relative humidity. Seed either were grown on petri dishes containing half-strength Murashige-Skoog medium (Sigma, St. Louis) supplemented with 2% sucrose and 0.7% agar or potted in soil (Metro-Mix 200; Sunagro).

RNA and DNA blot analyses.

Total RNA was extracted from 3-week-old plants with the TRI Reagents (Molecular Research Inc., Ohio, U.S.A.) according to the manufacturer's protocol. RNA (20 μg) was resolved on a 1% agarose gel containing 1.8% formaldehyde (Sambrook et al. 1989). Ethidium bromide was used to visualize the rRNA bands. For Southern blot analysis, genomic DNA was digested with *PvuII* and *BamHI*, electrophoresed through a 1% agarose gel, and blotted onto nylon membranes ((Hybond Hybond-N; Amersham Biosciences, Buckinghamshire, U.K.). RNA and

DNA gel blots were hybridized with gene-specific, ^{32}P -labeled, single-stranded DNA probes amplified by PCR except that only one primer was used to synthesize the antisense strand. Primers used for amplification of the probe template were as follows: SNC1-1 (5'ATATGGAGATAGCTTCTTCTCTG) and SNC1-4 (5'AAGATCATATCCGGCCATAACTG) for *SNC1* and PR1-1 (5'CTTTGTAGCTCTTGTAGGTGCTCT) and PR1-2 (5'TCCTGCATA-TGATGCTCCTTATTGA) for *PR1*.

Plasmid construction and plant transformation.

A 7.8-kb *Pst*I genomic fragment containing the *SNC1* promoter and the coding region was cloned from BAC clone F5D3 (Arabidopsis Biological Resource Center, Columbus, OH, U.S.A.) into the binary vector pCambia1300 (Cambia, Canberra, Australia) to generate the *pSNC1::SNC1* construct.

A 6-kb genomic fragment containing the *SNC1* coding region and 3' untranslated region was cloned from the BAC clone F5D3 into the binary vector pGreen-0229 (Hellens et al. 2000) to generate the *p35S::SNC1* construct.

The *pSNC1::GUS* construct was generated by cloning a *SNC1* genomic fragment with 1,904 bp 5' of the translation start codon ATG to 427 bp 3' of ATG into the polylinker region of the binary vector PZP212 (Diener et al. 2000) in frame with the *GUS* gene cassette.

To construct the *pSNC1::SNC1:GFP* chimeric gene, a *Stu*I site was added to the genomic fragment of *SNC1* before the stop codon of *SNC1* via PCR. An *EcoRV-Stu*I fragment of this product was ligated in frame with GFP flanked by *Stu*I and *Pst*I sites to create a 3' *SNC1:GFP* construct. The *Pst*I-*EcoRV* fragment of the 5' *SNC1* from the *pSNC1::SNC1* construct and the *EcoRV-Pst*I fragment of 3' *SNC1:GFP* from the 3' *SNC1:GFP* construct were ligated to the *Pst*I site of pCambia1300 to generate the *pSNC1::SNC1:GFP* construct.

Agrobacterium tumefaciens strain GV3101 (Koncz and Schell 1986) carrying different constructs was used to transform *Arabidopsis* plants via floral dip transformation (Clough and Bent 1998).

Pathogen resistance assay.

Bacterial growth in *Arabidopsis* was monitored as described (Yang et al. 2006). *P. syringae* pv. *tomato* DC3000 was grown overnight on the King's B medium and resuspended at 10^8 CFU 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. The amount of bacteria in plants was analyzed at 1 h after dipping (day 0) and 3 days after dipping (day 3). The aerial parts of three inoculated seedlings were pooled for each sample and three samples were collected for each genotype at one time point. Seedlings were ground in 1 ml of 10 mM MgCl $_2$ and serial dilutions of the ground tissue were used to determine the number of CFU per gram of leaf tissues.

Analysis of GUS activity.

Histochemical detection of GUS activity was performed as described (Hua et al. 2001). Tissues for GUS staining were incubated in staining solution (50 mM sodium phosphate, pH 7.0, 10 mM EDTA, 2 mM 5-bromo-4-chloro-3-indoyl glucuronide, 1 mM potassium ferricyanide, and 1 mM potassium ferrocyanide) at 37°C overnight. After incubation, stained tissues were cleared of chlorophyll in an ethanol series.

Generation of NILs.

NILs of *pSNC1::GUS* were isolated from F2 or F3 progenies of crosses between *pSNC1::GUS/Col* lines and *bon1-1snc1-11* by PCR-based genotyping. Amplification by a T-DNA primer with a gene-specific primer indicates the presence of a mutant

T-DNA allele, whereas amplification by two gene-specific primers indicates the presence of a wild-type allele. Primers used for *snc1-11* were wild-type allele: SNC1-1 and SNC1-4, and T-DNA mutant allele: SNC1-1 and JMLB1 (5'GGCAAT CAGCTGTTGCCCGTCTCACTGGTG). Primers for *bon1-1* were wild-type allele: MIN5 (5'ACGTTGAAAAGGCACTG GTGTATG) and MINRIV319 (5'ATCGAATTCGTGGACAA GTTTACCGAGAC) and T-DNA mutant allele: MIN5 and LB5 (5'TGCTGATCCATGTAGATTTC).

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