In situ deletions reveal regulatory components for expression of an intracellular immune receptor gene and its co-expressed genes in Arabidopsis

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Abstract

Intracellular immune receptor NLRs are highly regulated transcriptionally and post-transcriptionally for balanced plant defense and growth. NLR genes often exist in gene clusters and are usually co-expressed under various conditions. Despite of intensive studies of regulation of NLR proteins, cis-acting elements for NLR gene induction, repression or co-expression are largely unknown due to a larger than usual cis-region for their expression regulation. Here we used the CRISPR/Cas9 genome editing technology to generate a series of in situ deletions at the endogenous location of a NLR gene *SNC1* residing in the *RPP5* gene cluster. These deletions that made in the wild type and the *SNC1* constitutive expressing autoimmune mutant *bon1* revealed both positive and negative cis-acting elements for *SNC1* expression. Two transcription factors that could bind to these elements were found to have an impact on the expression of *SNC1*. In addition, co-expression of two genes with *SNC1* in the same cluster is found to be mostly dependent on the *SNC1* function. Therefore, *SNC1* expression is under complex local regulation involving multiple cis elements and *SNC1* itself is a critical regulator of gene expression of other NLR genes in the same gene cluster.

Introduction

Plants have complex innate immune systems for defending themselves against invasions and propagation of a variety of pathogens. Numerous receptors, often hundreds per genome, are used for detecting pathogen and triggering downstream defense responses (Kemmerling *et al.*, 2011; Jacob *et al.*, 2013; Shao *et al.*, 2016). These receptors are divided into two groups: pattern recognition receptors (PRRs) and nucleotide-binding leucine-rich repeat (NLR) receptors (Spoel and Dong, 2012; Cui *et al.*, 2015; Couto and Zipfel, 2016; Yu *et al.*, 2017). PRRs recognize microbial-derived signals in the environment and are often located on plasma membranes, while the NLR recognize pathogen secreted effectors inside the plant cells and are named intracellular immune receptors. The molecules recognized by PRR and NLR are not necessarily distinct (Thomma *et al.*, 2011), and their triggered responses are recently shown to be intertwined (Ngou *et al.*, 2021;Yuan *et al.*, 2021). Plant NLR proteins contain highly conserved central nucleotide binding site (NBS), C-terminal leucine-rich repeats (LRRs), and N-terminal domains (Takken and Goverse, 2012; Ngou *et al.*, 2021). The protein activity of NLR is tightly regulated. Pathogen effectors activate NLR by causing its conformational switch from ADP binding to ATP binding (Qi and Innes, 2013; Burdett *et al.*, 2019). Recent reports indicate that activation of at least three NLR proteins involves oligomerization: ZAR1 (HOPZ-ACTIVATED RESISTANCE 1) (Wan *et al.*, 2019), Roq1 (Recognition of XopQ 1) (Martin *et al.*, 2020) and RPP1 (RECOGNITION OF PERONOSPORA PARASITICA 1) (Ma *et al.*, 2020). Protein stability of NLR is also tightly regulated, as seen in their degradation by E3 ligases (Cheng *et al.*, 2011; Gou *et al.*, 2012). NLR genes are also highly regulated at the transcript level. Most of them are expressed at low levels in the absence of pathogens (Tan *et al.*, 2007). A survey showed that 75 out of the 124 NLR genes in *Arabidopsis thaliana* (referred as Arabidopsis thereafter) are up-regulated in response to at least one pathogen infection (Mohr *et al.*, 2010). Recent meta-analysis revealed that more than half of the NLR genes in the Arabidopsis Col-0 accession has increased expression after infection with *Pseudomonas syringae pv tomato* (*Pst*) DC3000 (Yang *et al.*, 2021). This is thought to enable effective defense response when pathogen is present but not compromise plant growth in the absence of pathogen. Un-controlled high expression of NLR genes is associated with constitutive activation of defense responses; and this autoimmunity has negative impacts on plant growth leading to lesion, dwarfism and even lethality (Gou and Hua, 2012; van Wersch *et al.*, 2016; Wan *et al.*, 2021).

How expression of NLR genes is regulated at the transcript level is not well understood compared to their regulation at the protein level. Transposons have been found to impact expression of NLR genes nearby. For example, the PHD (plant homeodomain)-finger protein EDM2 (enhanced downy mildew 2) is a chromatin-associated factor controlling expression of the NLR gene *RPP7* (*RECOGNITION OF PERONOSPORA PARASITICA 7*) and its related members (Lai *et al.*, 2020). The NLR gene *SNC1* (*SUPPRESSOR OF NPR1 CONSITUTIVE 1*) has been used as a model for dissecting of NLR transcription regulation. It is closely related to and physically linked to another NLR gene *RPP4* (*Resistance to Peronospora parasitica 4*) which confers downy mildew resistance (Van Der Biezen *et al.*, 2002). Like many NLR genes, *SNC1* is induced by pathogen and salicylic acid (Yang *et al.*, 2021). Similar to half of NLR genes in Arabidopsis, *SNC1* is located in a gene cluster (Noel *et al.*, 1999) and has co-expression with other NLR genes in the same cluster including *RPP4* and *SIKIC2* (*SIDEKICK SNC1 2*) (Noel *et al.*, 1999; Yang *et al.*, 2021). *SNC1* is upregulated in a few autoimmune mutants and contributes to their autoimmunity (Gou and Hua, 2012). Studies of an autoimmune mutant *bon1-1* (*bonzai1-1*, referred as *bon1* hereafter) has yielded a number of transcriptional regulators of the *SNC1* gene. *BON1* encodes a calcium binding protein and it interacts with calcium pumps ACA10 (Autoinhibited Ca^{2+}-ATPase 10) and ACA8 (Autoinhibited Ca^{2+}-ATPase 8) to affect cellular calcium signals (Yang *et al.*, 2017). The loss of *BON1* function leads to increased expression of *SNC1* and consequently an increased disease resistance and severe dwarfism (Yang and Hua, 2004). Suppressor and enhancer screens of *bon1* related mutants have yielded factors in the upregulation or repression of *SNC1* expression. These include histone modification related factors HUB1 (HISTONE MONO-UBIQUITINATION 1), ATRX7 (ARABIDOPSIS TRITHORAX-RELATED 7), HOS15 (HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENES 15) (Zou *et al.*, 2014; Gou *et al.*, 2017; Yang *et al.*, 2020), chromatin remodeling factor CHR5 (CHROMATIN REMODELING 5) (Zou *et al.*, 2017), as well as TCP transcription factors and their interacting protein MOS1 (MODIFIER OF *snc1*) (Li *et al.*, 2010; Bao *et al.*, 2014; Zhang *et al.*, 2018). Co-induction of genes in the same gene cluster with *SNC1* was observed in *bon1* mutants and appeared to be similarly influenced by *CHR5* and *HUB1* (Zou, B *et al.*, 2014; Zou *et al.*, 2017). It has been postulated that co-expression could be achieved through regulation of large chromatin structure or RNA silencing mechanisms (Yi and Richards, 2007; Li *et al.*, 2009). Alternatively, genes in

the same cluster could have similar cis-acting elements (such as an enhancer) and thus enable co-regulation by trans factors.

Despite of the advances in the identification of trans-acting factors, cis-acting elements responsible for *SNC1* regulation are largely unknown. Transgenic study revealed that DNA sequences outside 1.9 kb upstream of the translation start site and 1 kb downstream of the stop codon have repression activity on *SNC1* expression (Li *et al.*, 2010). Reporter gene analysis indicates that the 1.9 kb upstream region contains the regulatory elements of autoimmune induction of *SNC1* but not the distal elements for repressing *SNC1* expression (Li *et al.*, 2010). The dissection of cis-acting elements by the conventional reporter gene study is made difficult by the loss of *SNC1* repression activity from its endogenous region when *SNC1* is presented as a transgene not at its endogenous location.

Here, we dissected transcriptional regulation of NLR gene expression by manipulating the cis elements of the *SNC1* locus in vivo using the CRISPR/Cas9 genome editing technology. A series of DNA fragment deletions were made in both upstream and downstream regions of *SNC1* in the wild-type Arabidopsis plants as well as the autoimmune mutant *bon1*. Some of these deletions were found to decrease or increase expression of the *SNC1* gene. Two transcription factors that could bind to these elements were shown to have an impact on the expression of *SNC1*. In addition, co-expression of *RPP4* and *SIKIC2* with *SNC1* was found to be mostly dependent on *SNC1*. These results indicate that *SNC1* expression is under complex local regulation and *SNC1* itself is a critical regulator of gene expression in the *RPP5* gene cluster.

Materials and Methods

Generation of transgenic plants containing CRISPR/Cas9 constructs

Spacer sequences for guideRNAs (gRNAs) were searched in the region of interest using the website https://www.genome.arizona.edu/crispr/CRISPRsearch.html. Constructs were made according to the system utilizing two gRNAs as described (Xing *et al.*, 2014). In brief, PCR fragment was amplified from pCBC-DT1T2 with two long primers. The purified PCR fragment (T1T2-PCR), together with pHEC401 binary vector, were used for restriction-ligation reactions and transformed to *E. coli* and then *Agrobacterium* strain GV3101. The Arabidopsis wild-type Col-0 plants and the single mutant *bon1* plants were transformed with Agrobacteria containing the CRISPR/Cas9

construct via floral dipping method. Seeds collected from the transformed plants were selected on MS plates containing 25 mg/L hygromycin.

Genomic DNA was extracted from T1 transgenic plants. Gene-specific primers flanking the CRISPR target sites were used to amplify the genomic DNA by PCR and to identify putative deletion mutations. Mutations were verified by sequencing the PCR products from the putative mutants. T2 plants were crossed to Col-0 and *bon1* to obtain Cas9 free plants with homozygous deletion mutations in the F2 generation.

Spacer sequences used in this study and off-target potentials are listed in Table S1. Oligos used for cloning and genotyping are listed in Supporting Information Table S2.

Gene expression analysis

Leaf tissues were collected from 15-day-old plants grown under constant light. Total RNA was extracted with TRIzol reagent (Invitrogen) according to the manufacturer's protocol. The cDNA synthesis was performed by AffinityScript QPCR cDNA synthesis kit (Agilent Technologies). The Real-time quantitative PCR was carried out using the CFX96 real-time PCR system (Bio-Rad) using iQSYBR GREEN SuperMix (Bio-Rad). Relative expression of each gene was normalized to the expression of *ACTIN 2* in the same cDNA sample. The relative expression level was calculated by the $2^{-\Delta\Delta Ct}$ method using the CFX Manager Software, version 1.5 (Bio-Rad). Details of the oligos used for qPCR are in Supplementary Table S1.

Measurement of growth phenotypes

Fresh weight was measured by rosettes from 12-day-old plants grown under constant light condition or 20-day-old plants under the short-day condition (12 hr/12 hr). The dimeter of the rosette was measured using top-view photographs of the plant and defined as the diameter of the smallest circle that covers the whole rosette using ImageJ software. Thirty plants were measured for each genotype/condition.

ChIP-qPCR assay

Chromatin-immunoprecipitation (ChIP) experiment was performed as described in Lee et al. (2017) and Wang et al. (2021) with minor modifications. The coding sequence of LHY1 and bHLH28 was amplified from Col-0 cDNA and genomic DNA, respectively. PCR products were cloned into pCR8 vector (Invitrogen, K250020) and then

recombined into pSATN1 vector by LR clonase (Invitrogen, 11791020). The expression vectors were transformed into protoplast prepared from Col-0 seedlings. Primers used for vector construction and qPCR are listed in Table S2.

Statistical analysis

Data of gene expression and rosette morphology (weight and diameter) were subjected to a one-way analysis of variance (ANOVA) followed by Duncan's new multiple range test to assess differences between different genotypes. Significance was defined by *p* value as stated.

Results

Generation of in situ deletion series in the *SNC1* locus by CRISPR-Cas9 method

To identify regulatory cis-acting elements for the expression of *SNC1*, we generated a series of deletions in the *SNC1* locus by the two-target design CRISPR/Cas9 system (Xing *et al.*, 2014). A total of 14 PAMs (protospacer-adjacent motifs) were identified in the regions of 2 kb upstream of the translation initiation site and 4 kb downstream of stop codon (Fig. 1). A pair of guide RNAs (gRNAs) containing specific spacer sequences for targeting were used for generating deletions between two PAM sites, and 15 two-target CRISPR constructs were transformed to the wild-type Col-0 (referred as wild type from now on) as well as the *bon1* mutant. Deletion of DNA fragments between two PAM sites were detected by PCR using primers flanking the two PAM sites in the T1 transgenic plants. A total of 10 constructs yielded expected deletion events, and at least 6 independent deletion lines were identified for each of these 10 constructs in the wild type and *bon1* background combined (Fig. 1b). Because fewer lines were generated in *bon1* due to its lower transformation efficiency, and deletion mutants generated in the wild type was crossed with *bon1* to generate additional deletion mutants in *bon1*. More than 4 lines of the same type of deletion as determined by PCR in each of the wild type or *bon1* were randomly chosen and they exhibited a similar morphological phenotype to each other. Together with the predicted low to medium off-target potentials of these spacers (Table S1), this indicates that the alteration of the phenotype was caused by the intended deletion mutation but not the off-target mutations. One line per deletion type in wild type or *bon1* was brought into Cas9-free and homozygous for deletion mutations by backcross and selection from the

F2 generation. These lines were sequenced to determine the precise deletion positions (Fig. 1b) and were used for subsequent molecular analysis.

In total, 8 types of deletion mutations were obtained in the upstream region of *SNC1* and 2 types of deletion mutations were obtained in the downstream region of *SNC1* in each of the wild type and the *bon1* mutant (Fig. 1b). Among these, C1 and C7 deletions removed the transcriptional start sites (TSS) of *SNC1*, C2 was 5' to TSS, C3 was 5' next to C2, and C4 was 5' next to C3. C5 was a larger deletion that consisted of C4, C6 and part of C3. C8, originally thought to be homozygous for the large deletion between two target sites because of the uniform phenotype in its progeny, turned out to be a heterozygous for two mutant alleles. The C8 allele contained a large deletion between the two target sites as intended, and the C8' allele contained one 9 bp deletion at the first target site and a 1 bp insertion at the second target site. For downstream deletion mutations, C9 was 3' to the end of the 3'UTR of *SNC1*, and C10 was 3' to C9 and covered the whole coding sequence of *AT4G16880* that encodes a leucine-rich repeat (LRR) family protein.

Deletions of the transcription initiation site abolished expression of *SNC1*

We first analyzed *C1* and *C7* mutants with deletions that spanned the transcription initiation site in the wild-type and the *bon1* background. Neither of the mutants in the wild-type background exhibited any visible difference from the wild type grown under constant light (Fig. 2a, Fig. S1). However, the double mutants *C7 bon1* and *C1 bon1* had large rosette and flat leaves in contrast to *bon1* which had small and crinkled leaves under constant light (Fig. 2a). The fresh weights of the two double mutants were also higher compared to the *bon1* mutant, to a wild-type level under both constant light (Fig. S1). Under short days, a similar suppression effect of C7 and C1 mutations on *bon1* defects was also observed (Fig. S2). This suggests that autoimmune defect of *bon1* was suppressed by these two mutations.

We further analyzed the expression of *SNC1* in the single mutants of *C7* and *C1* and their respective double mutants with *bon1*. In *C7* and *C1* mutants, the transcript of *SNC1* was reduced compared to that in the wild type as detected by qRT-PCR (Fig. 2b). Consistent with prior reports, *SNC1* was up-regulated in the *bon1* mutant compared with the wild type (Fig. 2b). Both *C7* and *C1* mutations reduced the expression of *SNC1* in *bon1* to the level of the wild type (Fig. 2b). Therefore, the C1 and C7 deletions wiped out the expression of *SNC1*, as expected from the removing the TTS of a gene.

Consistent with previous findings that *SNC1* function is important for autoimmunity in *bon1*, abolishing *SNC1* expression reverted the autoimmune phenotype of *bon1*. This also indicates that in situ deletions is feasible for finding important cis-acting elements of gene expression.

Deletions in the upstream region have different effects on *SNC1* expression

Mutants of six deletions that resided in the upstream region of *SNC1* were analyzed. None of these mutations, *C2*, *C3*, *C4*, *C5*, *C6* and *C8*, affected the growth phenotype of the wild type (Fig. 3a, b, c). However, they exhibited suppression, enhancement, or no effect on the growth of *bon1* (Fig. 3a, b, c). Similar effects of those mutations were observed in the Col-0 and *bon1* under short days (Fig. S2).

The double mutants *C3bon1* and *C4bon1* had a similar morphological phenotype to *bon1* (Fig. 3a), suggesting little effect of these mutations on *bon1*. This was supported by a quantification of rosette area and fresh weight (Fig. S1). Expression levels of *SNC1* in the *C3* and *C4* single mutants and their respective *bon1* double mutants were similar as those in the wild type and *bon1* respectively (Fig. 3d). This indicates that deletions of C3 and C4 have little effect on the *SNC1* expression.

The double mutants *C2bon1* and *C5bon1* had a closer to wild-type morphology compared to *bon1* (Fig. 3b). The two double mutants were both significantly larger than *bon1* as shown by their rosette sizes and fresh weights (Fig. S1). qRT-PCR revealed that *SNC1* expression was significantly lower in the *C2bon1* and *C5bon1* double mutants than in the *bon1* mutant (Fig. 3e). Interestingly, *SNC1* expression was also reduced in the *C2* and *C5* single mutants compared to the wild type (Fig. 3e).

The double mutants *C8bon1* and *C6bon1* showed smaller size and more severe growth defects compared to *bon1*, with *C6bon1* showing a stronger growth defect (Fig. 3c). This was supported by a quantification of rosette size and fresh weight of the double mutants (Fig. S1). qRT-PCR revealed that *SNC1* transcript was higher in the *C8bon1* and even higher in *C6bon1* double mutants than *bon1* (Fig. 3f). *SNC1* also had an increased expression in the single *C8* mutant compared to the wild type, while it had a similar level in the single *C6* mutant compared to the wild type (Fig. 3f).

Therefore, C2 deletion greatly reduces *SNC1* expression in wild type and *bon1*, C5 deletion greatly reduces *SNC1* expression in *bon1*, C8 mutation (C8, C8' or C8 C8') moderately enhances *SNC1* expression in wild type and *bon1*, while C6 deletion greatly enhances *SNC1* expression in *bon1*.

Two deletions in the downstream region have opposite effects on *SNC1* expression

Two deletions 3' to the 3' UTR of *SNC1* were analyzed for their effects on autoimmunity and *SNC1* expression. These two single deletion mutants *C9* and *C10* showed no obvious morphological difference from the wild type (Fig. 4a). The *C9 bon1* double mutant displayed a larger rosette size while *C10bon1* displayed a smaller size compared to the *bon1* mutant (Fig. 4a). Quantification of rosette size and fresh weight supported these effects (Fig. S1). The *C9* and *C10* mutations had a similar effect under the short day and the long day conditions (Fig. S2). Correlated with the growth phenotypes, *SNC1* expression was lower in the *C9bon1* mutant but was higher in the *C10bon1* mutant compared to *bon1* (Fig. 4b). Interestingly, *SNC1* expression was lower in the *C9* mutant but higher in the *C10* single mutants compared to the wild type (Fig. 4b). These results suggest that C9 is a positive cis-acting element and C10 is a negative cis-acting element for *SNC1* in both the wild type and *bon1*.

Functions of two adjacent genes do not contribute to *SNC1* expression regulation

Because deletions of C1, C3, C4, C5, C6, and C8 resided in the gene *AT4G16892* and the C10 deletion removed the coding sequence of *AT4G16880*, their impact on *SNC1* expression could result from the loss of the function of the two genes or the cis-acting elements of *SNC1*. To differentiate these two senarios, we obtained the best possible loss of function mutants of these two genes available from the Arabidopsis Resource Center (Fig. 5a). The WiscDsLox504D04 mutant line had a T-DNA insetion in the last exon of *AT4G16892,* while the SALK_112108 had an insertion in the second exon of the gene *AT4G16890* (Fig. 5a). qRT-PCR analysis revealed that no expression of these two genes were detected in their respective mutants (Fig. 5b). Together with the insertion in exons, this data indicates that these two genes have lost their protein function if any in their respective mutants. These two mutants did not exhibit any significant morphological difference from the wild type (Fig. 5c). Double mutants between *bon1* and each of the mutants of *AT4G16880* and *AT4G16892* were generated by crossing. No morphological difference was observed among *bon1* and two *bon1* double mutants (Fig. 5c), indicating that functional disruption of the two genes is not the cause of the altered *SNC1* expression in these deletion mutants.

316 ***RPP4* and *SIKIC2* exhibited a similar expression pattern with *SNC1* in the deletion**
317 **mutants in *bon1***

318 We analyzed expression of *RPP4* and *SIKIC2* in the deletion mutants to assess
319 the effect of cis-acting elements of *SNC1* on other NLR genes in the same gene cluster.
320 These two genes were chosen because they have relatively high expression among NLR
321 genes in the cluster and therefore their expression can be more easily determined. *RPP4*
322 is the next NLR gene at the 3' end of *SNC1* and *SIKIC2* is several NLR genes away at
323 the 5' of *SNC1* in the cluster. Both *RPP4* and *SIKIC2* had a higher expression in the
324 *bon1* mutant than in the wild type (Fig. 6a). Similar to *SNC1*, *RPP4* had a lower
325 expression in each of the *C5bon1*, *C1bon1*, *C7bon1*, *C2bon1*, and *C9bon1* double
326 mutants than in *bon1* (Fig. 6a). Also similar to *SNC1,* it had a higher expression in
327 *C8bon1*, *C6bon1*, and *C10bon1* double mutants than in *bon1* (Fig. 6b). Therefore, the
328 effects of deletions of these cis-acting elements were the same for *RPP4* and *SNC1*.
329 These deletions also had an impact on the expression of *SIKIC2* in *bon1* similar to the
330 expression of *SNC1* (Fig. 6a). Interestingly, the C3 and C4 deletions that did not
331 significantly affect *SNC1* expression did not affect the expression of *RPP4* or *SIKIC2*
332 in the *bon1* mutant either. The only exception is a higher expression of *RPP4* in *C4*
333 *bon1* compared to *bon1* while *SNC1* was expressed at a comparable level in *C4bon1*
334 and *bon1*.

335 The impact of these deletions on the expression of *RPP4* and *SIKIC2* was also
336 examined in the *BON1* wild-type background. The two deletions (C8 and C10) that
337 increased expression of *SNC1* also increased expression of *RPP4* and *SIKIC2* (Fig. 6b).
338 For deletions that reduced *SNC1* expression in the wild type, the effects on *RPP4* and
339 *SIKIC2* were complex. *RPP4* had a reduced expression in the mutants of *C1*, *C7*, *C5*
340 and *C9*, but increased level in the *C2* mutant (Fig. 6b). *SIKIC2* had a reduced or similar
341 expression level in the *C1* and *C5* mutants but a higher level in the *C7*, *C2*, and *C9*
342 mutants (Fig. 6b). Therefore, in the wild-type background, deletions that reduce *SNC1*
343 expression reduced expression of *RPP4* and *SIKIC2* for *C1* and *C5*; reduced *RPP4* but
344 increased *SIKIC2* for C7 and C9; and increased *RPP4* and *SIKIC2* for C2. However,
345 the expression of these genes in the wild type was very low, and the expression
346 difference, if any, was less than 2 folds. Additional biological repeats are needed to
347 verify expression in the wild-type background.

348

349 ***SNC1* and *PAD4* are both required for upregulation of *RPP4* and *SIKIC2* in *bon1***

The high expression correlation of *SNC1* with *RPP4* and *SIKIC2* in the deletion mutants of the *SNC1* region suggests that the *SNC1* expression level might determine the expression of the other two NLR genes. The other hypothesis that these elements all have a long-distance effect on the expression of distant genes seems to be less likely. To further test the hypothesis that the *SNC1* gene function rather than the cis-DNA element has a major function in co-expression, we compared the effect of loss of function mutations of *pad4* and *snc1* on the expression of *RPP4* and *SIKC2*. In both *bon1pad4* and *bon1 snc1-11* double mutants, expression of both *RPP4* and *SIKIC2* were reduced from a high level in *bon1* to the wild-type level (Fig. 7b-c). Interestingly, the expression of *RPP4* was reduced by both mutations while *SICK2* was reduced by only the *snc1-11* mutation in the *BON1* wild-type background (Fig. 7b-c). This indicates that the effect of *SNC1* has an effect as great as if not greater than *PAD4* in the regulation of *RPP4* and *SIKIC2* expression.

Loss of LHY or bHLH28 function reduces expression of *SNC1*

Because the deletions in the 1.5 kb 5' of the transcription initiation site affect *SNC1* expression, we looked for transcription factors that might bind to this region and promote *SNC1* expression. An early study has identified binding sites of 527 of transcription factors across genome by DAP-seq (O'Malley *et al.*, 2016), and therefore we searched this data set for proteins that have significant bindings to this region. A number of transcription factors including several bHLH (basic helix-loop-helix) factors were identified in the C2 and C5 regions where positive cis-acting elements reside. We assessed the function of two of these proteins, LHY1 (LATE ELONGATED HYPOCOTYL 1) and bHLH28, in the regulation of *SNC1* gene expression. These two proteins had the highest binding signal among bHLH proteins at C2 and C5 regions respectively (Fig. 8a).

We isolated a T-DNA insertion mutant for each of the *LHY* and *bHLH28* genes. These two mutants were loss of function mutants because they had T-DNA inserted in the coding region and no expression of these genes was detected in their respective mutants (Fig. S3). These two mutations were each introduced to *bon1* by crossing. The double mutants *bhlh28 bon1* and *lhy1 bon1* were both larger in size compared to the *bon1* single mutant (Fig. 8b), indicating a partial suppression of the *bon1* defect.

We further analyzed the expression of *SNC1* in the single mutants of *bhlh28* and *lhy1* as well as their respective double mutants with *bon1*. *SNC1* was expressed at a

similar level in the single mutants and the wild type as detected by qRT-PCR (Fig. 8c). However, the expression of *SNC1* in both *bhlh28bon1* and *lhy1bon1* was significantly lower than that in *bon1* with the *lhy1* mutation having a larger impact than the *bhlh28* mutation (Fig. 8c). Therefore, *bHLH28* and *LHY1* both promote expression of *SNC1* in the *bon1* mutant.

To verify the direct binding of LHY1 and bHLH28 proteins at the *SNC1* promoter observed in the DAP-seq, we performed additional ChIP-qPCR analysis using transient expression of LHY1-GFP and bHLH28-GFP fusion proteins in protoplasts prepared from wild type Col-0 seedlings. We chose "A" site and "B" site at *SNC1* promoter region for detecting the direct binding of LHY1 and bHLH28, respectively (Fig. 8a). A region 'N' at *SNC1* gene body without any binding signals of the two proteins in the DAP-seq database was included in the analysis as a control. Both LHY1-GFP and bHLH28-GFP fusion proteins were localized in the nucleus as expected when expressed in Arabidopsis protoplasts (Fig. S3c). Enrichment of LHY1 and bHLH28 binding (antibody versus no antibody) was observed at the A and B site, respectively, in two independent biological repeats, while no binding was observed in the control site N for either of the proteins (Fig. 8d). These data further support that LHY1 and bHLH28 promote *SNC1* transcription in *bon1* mutant by directly binding to the *SNC1* promoter region.

Discussion

Here we report the use of genome editing tool to directly assess the impact of cis-acting elements in the regulation of a NLR gene *SNC1*. We identified several potential cis DNA elements at the *SNC1* locus that have either positive or negative impact on *SNC1* gene expression. Furthermore, we identified two transcription factors that could bind to the *SNC1* region and impact the expression of *SNC1*. In addition, the impact of deletion of these elements around the *SNC1* gene is analyzed on the expression of two *SNC1*-coexpressed NLR genes in the same gene cluster. Co-induction of these three genes in *bon1* is largely dependent on the function of *SNC1*, suggesting a co-regulation mechanism via *SNC1*. This study thus enhances our understanding of the complex transcriptional regulation of the NLR gene *SNC1* and emphasizes an important role of *SNC1* in expression regulation of other NLR genes.

New tools to identify cis-acting elements for transcriptional regulation

The identification of cis-acting elements has been traditionally done by transgene approach, that is, the regulatory elements are assayed as a transgene. This approach, although very effective in most cases, has its limitations. One is that random integration could potentially bring in enhancer or repressors at the transgene integration site to impact transgene expression. The other is the difficulty in determining the size of the fragment that encompasses all regulatory elements for gene expression. Sometimes, a larger region of the DNA sequence (such as heterochromatin region) might be needed for gene expression regulation (Brumos *et al.*, 2020).

Genome editing makes it possible to modify sequence elements in situ for identifying cis-acting elements important for transcriptional regulation. These on-site modifications preserve all other factors such as transacting factors, chromatin structures and distal cis-acting elements as identical as possible to their native status. Genome editing has been widely used for editing gene function, but not yet for cis-element identification. Here we used two-target scheme of CRISPR/Cas9 technology to create 10 in situ genomic DNA deletions of 179 bp to 2500 bp with medium size of 750 bp (Fig. 1b). A large region of several kb can therefore be covered by several deletions for assessing boundaries of cis-acting elements of any genes. Small deletions can also be made to assess the function of refined elements in gene expression regulation. Indeed, this technology has been used in refine the expression of tomato yield genes (Rodríguez-Leal *et al.*, 2017).

However, the CRISPR/Cas9 system used in this study has limitations due to the availability of PAM sites. It is not possible to generate deletions at specific sites for refined deletions. With the development of new and modified CRISPR systems, more targeted editing including deletions can be made, which will enable the identification of more refined elements and ultimately to single base pair precision.

Cis-acting elements for *SNC1* expression

We have identified several potential positive and negative cis-acting elements for the expression of *SNC1* (Fig. 9). The impact of the deletions or insertion (as in one of the C8 mutations) most likely results from a loss of binding sites for a trans-acting factor (such as a transcriptional activator or repressor), but it may also result from an altered proximity of another trans-acting factor binding site with TSS or a change of local chromatin structure. If we apply the simplest hypothesis (that all cis-acting elements function only as trans-acting factor binding sites and the cis-acting elements

work independently of each other) to the deletion defined elements, we can propose a mosaic pattern with positive, negative, and neutral elements spanning the upstream region of *SNC1* as well as one positive element and one negative element in the downstream of *SNC1*. This simplified view could be tested by more refined deletion mutants in the locus. Alternative models could be proposed where the deletion might cause chromatin structure change but not necessarily the loss of transcription factor binding sites. New chromatin structure might form and/or distal trans-acting factors might gain new protein interactions with large deletions. This can be tested by analyzing features of chromatin in the mutants.

In sum, this study has identified C2 and part of C5 in the upstream region and C9 in the downstream region as potential positive cis-acting elements for both basal and induced *SNC1* expression (Fig. 9). Part of C8 in the upstream region and C10 in the downstream region are likely responsible for basal and induced *SNC1* expression while C6 is likely a negative cis-acting element of induced *SNC1* expression in *bon1* (Fig. 9). The distinction between *bon1*-induced expression only and constitutive (both basal and *bon1* induced) is not very strict, because C2, C5, and C8 deletions have a slightly larger effect in *bon1* than in wild type. Generation of additional deletions with smaller sizes will reveal a more precise picture of the cis-acting elements.

Two transcription factors affect *SNC1* expression

We identified two transcription factors that impact *SNC1* expression. These two proteins directly bind to the *SNC1* region in vivo in the ChIP-qPCR experiment (Fig. 8d), and the expression of *SNC1* in *bon1* is reduced by the loss of their function (Fig. 8c). These data suggest that they are direct transcription factors of *SNC1* that binds to the cis-acting elements of the *SNC1* and affects its transcription. Nevertheless, the identification of these two potential factors opened new windows for investigating regulation of NLR gene expression. The first transcription factor identified is a circadian clock gene *LHY* that is involved in feedback loop for clock activity regulation (Alabadí *et al.*, 2001). Besides its crucial function in circadian regulation, the *lhy* mutants were shown to affect resistance to both bacterial and oomycete pathogens (Zhang *et al.*, 2013). The regulation of *SNC1* expression by *LHY* suggests that NLR gene regulation could contribute to the role of *LHY* in defense response regulation. The second factor identified for SNC1 expression is the *bHLH28* gene. Also named as MYC5, it binds to JAZ promoters and has overlapping functions with MYC2, MYC3

and MYC4 to control some of the early JA-responsive genes (Song *et al.*, 2017). The *myc5* mutant was shown to be more susceptible to necrotrophic pathogen but more resistant to bacterial pathogen *Pst* DC3000 likely through stomatal defense (Song *et al.*, 2017). Here we find that the *bhlh28* mutation reduces the expression of *SNC1*, which would likely result in reduced disease resistance. Therefore, *bHLH28* may have different target genes that impact defense responses in different processes.

Understanding of transcriptional control of NLR genes have large implications in generating disease resistance crop plants. Introducing multiple NLR genes (sometimes in a gene cluster) is an effective way to achieve broad spectrum and durable disease resistance (Monteiro and Nishimura, 2018; Van de Weyer *et al.*, 2019). As a higher than wild-type expression of NLR genes as transgenes is a common phenomenon, it is important to understand their transcriptional regulation to prevent autoimmunity in the process of transferring NLR genes. This study reveals complex regulation of NLR gene expression through cis-acting elements and demonstrates of the use of genome editing for cis-element identification. The refinement of cis-acting elements through genome editing techniques and a further understanding of expression of genes in a gene cluster which will have significant implication in generating disease resistant plants using NLR genes.

Co-expression of NLR genes in the *RPP5* gene cluster

Co-expression has been observed for NLR genes in the Col-0 *RPP5* gene cluster including *SNC1*, *RPP4* and *SIKIC2* (Yi and Richards, 2007; Dong *et al.*, 2018). Here we found that increased expression of *RPP4* and *SIKIC2* is dependent on increased expression of *SNC1* (Fig. 6), because C2 and C5 deletions that abolished *SNC1* expression also reduced expression of *RPP4* and *SIKIC2* while C6 and C10 deletions that increased *SNC1* expression also increased expression of *RPP4* and *SIKIC2* in the *bon1* background. In addition, deletions C8 and C10 that increased *SNC1* expression in wild-type background also increased expression of *RPP4* and *SIKIC2* (Fig. 6). Therefore, *SNC1* upregulation due to local cis-element changes is sufficient to induce higher expression of *RPP4* and *SIKIC2*. The dependence of *RPP4* and *SIKIC2* expression on the *SNC1* expression level is further supported by their dependence on the *SNC1* function. The loss of *SNC1* function reduced the higher expression of these two genes in *bon1* to the wild-type level (Fig. 7) and its effect is similar to that of the

loss of function of *PAD4*. Together, they indicate that *SNC1* function is critical for the co-induction of NLR genes in the *RPP5* cluster.

In addition to the function of regulating other NLR genes*SNC1* also has a feedback regulation on its own expression (Yang and Hua, 2004). It is possible that a higher expression of *SNC1* triggers accumulation of SA, production of trans factors or change of chromatin structures which feed forward on the expression of itself and other NLR genes (Fig. 10a). Alternatively, *SNC1* protein accumulation itself is responsible for the upregulation of *RPP4* and *SIKIC2* (Fig. 10b). *SNC1* is capable to physically interact with transcriptional regulators TPR protein (Zhu *et al.*, 2010) , suggesting a direct function in transcriptional regulation. We do not have evidence to distinguish between these two possibilities yet. The reduced basal *SNC1* expression correlates in general with a decreased *RPP4* expression but often does not correlate with the expression of *SIKIC2* (Fig. 6). This suggests that basal *SIKIC2* expression is not dependent on *SNC1* but *RPP4* might be. Whether or not basal expression of *SNC1* affects basal SA or other signaling molecules is yet to be determined.

Data availability

Data are included in the manuscript or in supplemental materials.

Author Contribution

JH conceived the project, JH and TZ supervised the project, HY, LY, ZL, BL, SG, FS, and YW conducted the experiments, HY and JH analyzed the data and wrote the manuscript with inputs from all authors.

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Figure Legends

Figure 1. Deletion mutants generated by CRISPR-Cas9 editing.

a. Diagram of the *SNC1* region and positions of deletions. The gene *AT4G16892* is in the 5' upstream of *SNC1* and the gene ATG16880 is in the 3' of the *SNC1*. Target sites of CRISPR-Cas9 are shown as gray vertical lines. Horizontal lines indicate deleted regions, and the name of deletions are showed as from C1 to C10. "*" indicates mutations in C8'. Gray boxes are coding regions. TSS, transcription start site.

b. Information of deletion mutant lines generated in Col-0 and *bon1*. Listed in the table is the name, starting point, ending point and length of deletions as well as the number of mutant lines generated in the wild type and *bon1*.

Figure 2. Removing transcription initiation site abolishes expression of *SNC1*.

(a) Growth phenotypes of C1 and C7 deletion mutants in the wild type and *bon1*. (b) Relative *SNC1* expression in the C1 and C7 deletion mutants in *bon1* and the wild-type Col-0 assayed by qRT-PCR. Actin was used as a reference gene and relative expression is compared to that of Col-0. Shown are average of three biological repeats and error bars indicate standard deviations. Different letters indicate statistically significant differences between genotypes by one-way analysis of variance (ANOVA) (p<0.05)..

Figure 3. Effect of deletions in the upstream region on *SNC1* expression.

(a, b, c) Growth phenotypes of deletion mutants of C3 and C4 (a), C2 and C5 (b), C6 and C8 (c) in the wild-type Col-0 and *bon1*. A representative line is shown here. (d, e, f) Relative *SNC1* expression in deletion mutants of C3 and C4 (d), C2 and C5 (e), as well as C6 and C8 (f) in *bon1* and the wild type assayed by qRT-PCR. Shown are average of three biological repeats and error bars indicate standard deviations (SDs). Different letters indicate statistically significant differences between genotypes by one-way analysis of variance (ANOVA) (p<0.05)..

Figure 4. Effect of deletions in the downstream region on *SNC1* expression.

(a) Growth phenotypes of C9 and C10 deletion mutants in the wild type and *bon1*. (b) Relative *SNC1* expression in the C9 and C10 deletion mutants in *bon1* and the wild-type Col-0 assayed by qRT-PCR. Actin was used as a reference gene and expression levels was compared to the Col-0. Values are means of three biological repeats and error bars indicate standard deviations. Different letters indicate statistically significant differences between genotypes by one-way analysis of variance (ANOVA) (p<0.05)..

Figure 5. Impact of loss of function of two adjacent genes on *SNC1* expression.
(a) Diagram of the gene structure of *At4g16892* and *At4g16880* and the T-DNA insertion sites of the mutants. (b) The qPCR analysis of *At4g16892* and *At4g16880* transcript level in wild type Col-0 and their respective T-DNA insertion mutants. ACTIN2 was used as an internal control. "*" indicate statistically significant differences between genotypes (p<0.05, student's t test). (c) Growth phenotype of double mutants of *BON1* with each of two genes.

Figure 6. Expression of *RPP4* and *SIKIC2* in the deletion mutants.
Relative expression of *SNC1, RPP4,* and *SIKIC2* in 10 deletion mutants in *bon1* (a) and wild type (b). Actin was used as a reference gene and shown are expression relative to that in the wild type (set as 1) for each of the three genes. Values are means of three biological repeats, and error bars indicate standard deviations. Different letters indicate different levels of expression among different genotypes for *SNC1* (without '), *RPP4* (with ') and *SIKIC2* (with ' ') at p<0.05 by one-way analysis of variance (ANOVA). Blue, red, and yellow ovals indicate reduced, increased and same expression in the deletion mutants compared to their corresponding wild-type Col-0.

Fig 7. Expression of *SNC1, RPP4* and *SIKIC2* in the *snc1* and *pad4* mutants.
Relative expression of *SNC1* (a)*, RPP4* (b)*,* and *SIKIC2* (c) in *pad4* and *snc1-11* mutants in the *BON1* wild-type and mutant background. Actin was used as a reference gene and shown are expression relative to that in the wild type (set as 1) for each of the three genes. Values are means of three biological repeats, and error bars indicate standard deviations. Different letters indicate different levels of expression among different genotypes at p<0.05 by one-way analysis of variance (ANOVA) .

Figure 8. Function of *LHY1* and *bHLH28* in *SNC1* expression regulation.
(a) Binding of two transcriptional factors LHY1 and bHLH28 in the *SNC1* locus from DAP-seq database. This is a redrawn from the browser image. Binding is shown by colored blocks with height represent the level of binding detected. (b) Growth phenotype of the mutants *bhlh28* and *lhy1* in the wild type or *bon1*. Plants were grown under 16h/8h light at 22°C. (c) Relative *SNC1* expression in the *bhlh28* and *lhy1* single mutants and double mutants with *bon1* assayed by qRT-PCR. Actin was used as a reference gene and expression levels was compared to the Col-0. Shown are average of

three biological repeats and error bars indicate standard deviations. Different letters indicate statistically significant differences between genotypes ($p<0.05$, student's t test). (d) ChIP-qPCR analyses of LHY1-GFP and bHLH28-GFP to the *SNC1* promoter region. The binding of LHY1-GFP and bHLH28-GFP was detected at "A" site and "B" site (depicted in a), respectively. Data of two independent biological replicates were shown. "N" site (depicted in a) is a region at the *SNC1* gene body with no binding signal detected for LHY1 or bHLH28 in the DAP-seq database. "GFP" are samples incubated with anti-GFP antibody and "NoAb" are samples without anti-GFP antibody. Different letters indicate statistically significant differences between genotypes by one-way analysis of variance (ANOVA) ($p<0.05$).

Figure 9. Diagram of putative cis-acting elements and transcription factors at the *SNC1* locus.

Diagram of the genomic region of the *SNC1* gene. Color lines below the genomic diagram depict deletions used in this study. Color blocks above the *SNC1* locus diagram depict putative positive and negative cis-acting elements. Ovals represent the two transcription factors that potentially bind to the region. Red indicates a negative effect of the element on *SNC1* expression (its deletion increases *SNC1* expression) and blue indicates a positive effect on *SNC1* expression.

Figure 10. Potential mechanisms for co-induction of NLR genes in the *RPP5* gene cluster.

Shown is the diagram of the *RPP5* cluster that consists of *SNC1*, *RPP4, SIKIC2* and additional NLR genes. Expression of *RPP4* and *SICK2* and potentially other NLR genes in the cluster are increased when *SNC1* expression or function are induced. SNC1 may mediates the induction of other NLR genes through SA accumulation, trans-factors or chromatin structure changes (a) or through direct regulation on other NLR genes (b). *SNC1* itself is under a feedback amplification regulation. Arrows indicate positive regulation.

Supplemental information

Supple fig 1. Growth phenotypes of deletion mutants grown under constant light.

Supple fig 2. Growth phenotypes of deletion mutants grown under short day.

Supple fig 3. Characterization of *LHY1* and *bHLH28* genes.

Table S1. List of spacer sequences and their off-target potential.

Table S2. List of oligos used for genotyping, cloning and qPCR.

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