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DNA Repair Proteins Are Directly Involved in Regulation of Gene Expression during Plant Immune Response

Junqi Song,[1] Wendy E. Durrant,[1] Shui Wang,[1] Shunping Yan,[1] Ek Han Tan,[1] and Xinnian Dong[1,*]
[1]Department of Biology, Duke University, Box 90338, Durham, NC 27708, USA
*Correspondence: xdong@duke.edu
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SUMMARY

Systemic acquired resistance (SAR), an inducible plant-defense response to local infection, requires the signaling molecule salicylic acid (SA) and the transcriptional coactivator NPR1, with concerted activation of *pathogenesis-related* (*PR*) genes. *Arabidopsis sni1* is an *npr1* suppressor and derepression of defense genes in *sni1* causes reduced growth and fertility and increased homologous recombination. Characterizing suppressors of *sni1*, we identify the DNA damage repair proteins SSN2 and RAD51D as genetic and physical interactors with SNI1. During plant defense, SSN2 and possibly RAD51D replace the transcription repressor SNI1 at pathogenesis-related gene promoters. In the presence of SNI1, NPR1 is also required for SSN2 binding. Thus, coordinated action of SNI1, SSN2-RAD51D, and NPR1 ensures the tight control of plant immune gene expression. Given that the SSN2-RAD51D complex is conserved in eukaryotes, their dual function in homologous recombination and transcription regulation of plant-defense genes suggests a general link between these two stress responses.

INTRODUCTION

Plants are constantly exposed to a wide range of microbial pathogens in nature. They have evolved effective defense mechanisms to cope with infection (Jones and Dangl, 2006). Systemic acquired resistance (SAR) is an inducible defense response that occurs after local infection and provides long-lasting, broad-spectrum resistance to secondary infection (Durrant and Dong, 2004; Ryals et al., 1996). The onset of SAR requires local and systemic increases of the signal molecule salicylic acid (SA), accompanied by concerted activation of *pathogenesis-related* (*PR*) genes. These genes encode small secreted or small vacuole-targeted proteins with antimicrobial activities. In particular, *PR1* and *β-1,3-glucanase* (*BGL2*, also called *PR2*) are well-used molecular markers of SAR (Uknes et al., 1992).

Constitutive activation of defense is usually detrimental to plant growth and development and therefore both temporal and spatial expression of *PR* genes are strictly regulated at multiple levels. Mutants with elevated expression of defense genes often have decreased plant fitness associated with reduced stature, loss of apical dominance, and decreased fertility (Bowling et al., 1997; Dietrich et al., 1994; Greenberg et al., 1994). The inducible nature of SAR allows plants to activate defense only when the benefit of resistance outweighs the cost on growth and development. Previously in a genetic screen for *npr1* suppressors *sni1* was identified, which restored the inducibility of *PR* gene expression in the *npr1* background (Li et al., 1999). SNI1 encodes a leucine-rich nuclear protein and does not share any obvious homology with known proteins in the database. Structural similarity searches revealed that SNI1 may function as a scaffold for interaction with various signaling proteins (Mosher et al., 2006). However, identification of potential interactors with SNI1 by yeast two-hybrid analysis was hampered by its transcriptional repressor activity (Mosher et al., 2006). The underlying molecular mechanism of how SNI1 regulates defense response remains elusive.

In this study, we identified a suppressor of *sni1*, *ssn2*, which encodes a protein containing a SWIM (SWI2/SNF2 and MuDR) domain found in a variety of prokaryotic and eukaryotic proteins. We present compelling evidence that SNI1 forms complexes with both SSN2 and RAD51D, which are previously known partners in homologous DNA recombination (Durrant et al., 2007; Martín et al., 2006). SNI1 binds to the *PR1* gene promoter to suppress defense response in the absence of pathogen challenge and is removed in response to induction. Our data suggest that the positive regulators SSN2 and RAD51D are recruited to the *PR1* gene promoter through interaction with the TGA7 transcription factor and by the function of the transcription cofactor NPR1. Our findings reveal a previously uncharacterized function of the SSN2-RAD51D complex in direct transcriptional regulation and demonstrate that multicellular organisms employ these proteins to modulate their transcriptional responses to external stimuli.

RESULTS

SSN2, Suppressor of *sni1* 2, Is a Positive Regulator of Plant Defense

The identification of *rad51d* (*ssn1*) as a mutant compromised in plant-defense gene expression showed that a DNA repair protein can play a role in transcription (Durrant et al., 2007). To investigate whether this is an isolated association or there is a deeper connection between these two stress responses, we

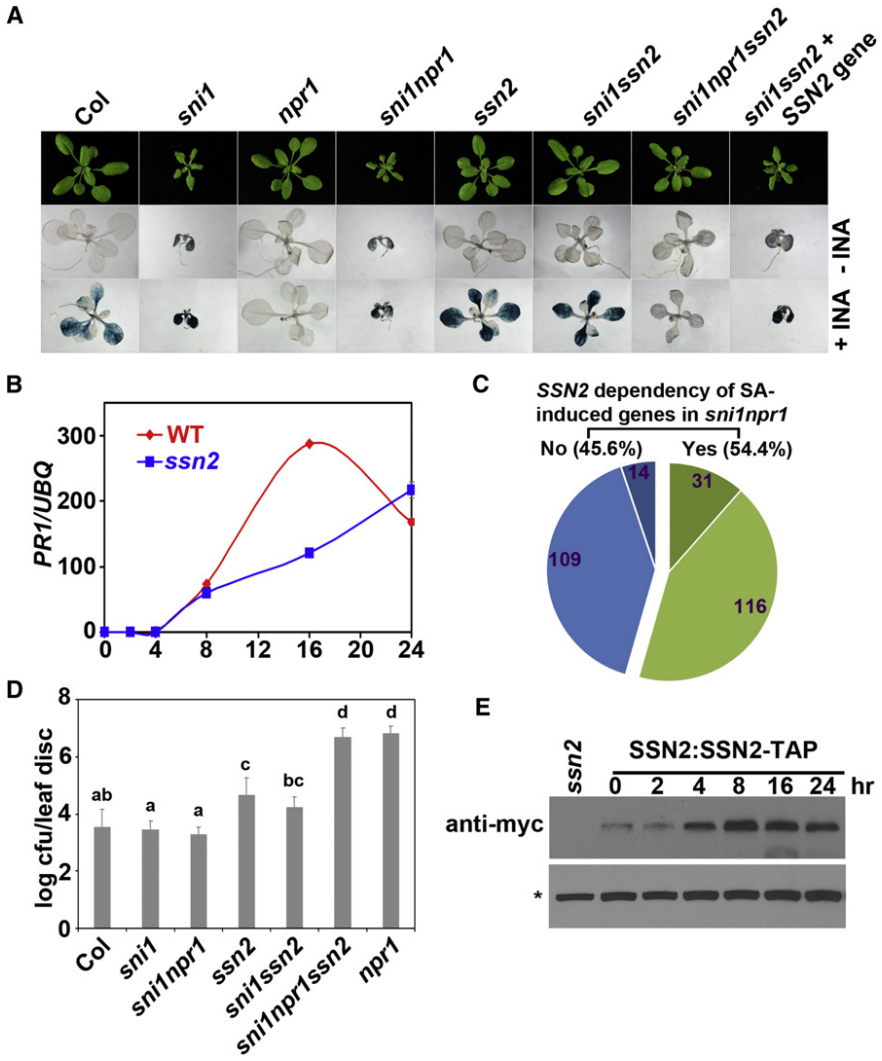

Figure 1. The *ssn2* Mutant Is a Suppressor of *sni1* and Is Compromised in Disease Resistance

(A) Morphological phenotypes of 3-week-old soil-grown plants and expression of *BGL2:GUS* in 2-week-old untreated plants grown on MS plates or plants treated with 50 μM INA.

(B) SA-induced *PR1* gene expression is compromised in the *ssn2-1* mutant. *PR1* gene expression was determined by real-time PCR and normalized with *ubiquitin* (*UBQ*). Error bars represent SE. The experiment has been repeated three times with similar results.

(C) Through two-way ANOVA analysis of the microarray data, 1143 genes were found to have significant interactions between genotypes (*sni1npr1* and *sni1npr1ssn2*) and treatments (water and SA) ($p < 0.01$). Among the 270 genes induced by SA in *sni1npr1* ($p < 0.05$), 147 genes (54.4%) were SSN2 dependent (green + shaded green) and 123 genes (45.6%) were SSN2 independent (blue + shaded blue). Among the SSN2-dependent genes, defense-response genes were significantly enriched (31 genes, 21.1%) (shaded green). The percentage is lower in the SSN2-independent genes (shaded blue).

(D) Three-week-old soil-grown plants were infiltrated with *Psm* ES4326 (OD_{600} = 0.0001) and colony-forming-units (cfu) were quantified 3 days after inoculation. Error bars represent 95% confidence limits of log-transformed data. The data were analyzed by Student's t test. Different letters indicate statistically significant differences between genotypes ($p < 0.05$). The experiments were repeated three times with similar results.

(E) SSN2 protein is upregulated by SA treatment. Three-week-old *SSN2:SSN2-TAP* (in *ssn2-1*) plants were treated with 0.5 mM SA. Proteins were extracted at the indicated times and detected with an anti-myc antibody. See also Figure S1 and Document S2.

studied another mutant, *ssn2,* isolated in the same genetic screen for suppressors of *sni1*. The *sni1* single mutant exhibits pleiotropic phenotypes, such as early flowering, reduced fertility, short roots, and narrow leaves. In the *sni1* mutant, several *PR* defense marker genes show elevated background expression (Li et al., 1999). The suppressor screen was designed to search for mutants that resembled wild-type in morphology and had abolished the background expression of *PR* genes in *sni1*. As shown in Figure 1A, *ssn2* restored wild-type morphology to both *sni1* and *sni1npr1*. The elevated background expression of the SA-responsive *BGL2:GUS* reporter in *sni1* and *sni1npr1* was also suppressed in *sni1ssn2* and *sni1npr1ssn2*. Moreover, in *sni1npr1ssn2,* the reporter gene lost its responsiveness to exogenous application of a SA functional analog INA (2,6-dichloroisonicotinic acid) (Figure 1A). A time course expression analysis performed on the *PR1* gene also showed a significant delay in gene induction in the *ssn2* single mutant (Figure 1B).

To determine whether the effect of *ssn2* is restricted to *BGL2:GUS* and *PR1* or general to the transcription reprogramming during plant defense, we performed microarrays on *sni1npr1* and *sni1npr1ssn2* with and without SA treatment. As shown in Figure 1C, among the 270 genes significantly induced in *sni1npr1* (p value < 0.05), 147 (54.4%) were dependent on SSN2 (Document S2, available online). Gene ontology analysis found 31 defense-related genes in the SSN2-dependent group (21.1%) and 14 in the SSN2-independent group (11.4%). We also performed qPCR on nine well-known defense genes detected in the microarray and found that SA induction of genes such as *PR1, PR2, PR5, EDS1,* and *PAD4* was diminished by the *ssn2* mutation (Figure S1A).

The *ssn2* mutation was mapped to the At4g33925 locus (Figure S1B). Through sequencing analysis, a 3.4 kb *gypsy*-like retrotransposon insertion was identified in the first exon of At4g33925 in *ssn2-1* (Figures S1C and S1D). A ~40 kb deletion and a large DNA rearrangement were found in the chromosomal region of At4g33925 for *ssn2-2* and *ssn2-3*, respectively. The *ssn2-1* allele was selected for further experiments and is hereafter referred to as *ssn2*. To confirm that At4g33925 is *SSN2*, we transformed the genomic DNA containing the entire At4g33925 gene into *sni1ssn2*, and the resulting homozygous transgenic lines restored the *sni1* morphology and the background *BGL2:GUS* expression (Figure 1A).

SSN2 encodes a small protein with one recognizable domain known as the SWIM domain, which has been shown to be involved in both DNA binding and protein-protein interaction in different proteins (Makarova et al., 2002; Martín et al., 2006). Homologs of SSN2 have been found in all eukaryotes (Martín et al., 2006). Confocal fluorescence microscopic examination of transgenic plants expressing a functional *35S:SSN2-GFP* transgene showed that SSN2 was predominantly localized in the nucleus (Figure S1E), similar to SNI1 (Mosher et al., 2006). By using a fusion of the *SSN2* promoter to the *GUS* reporter, *SSN2* gene expression was detected in leaves, predominantly in shoot apexes. Moderate expression of *SSN2* was also observed in roots (Figure S1F).

To evaluate the biological function of SSN2 in plant-defense response, we challenged the *ssn2* mutant with *Pseudomonas syringae* pv. *maculicola* ES4326 (*Psm* ES4326). As shown in Figure 1D, there was an increase in growth of *Psm* ES4326 in the *ssn2* single mutant. This defect in resistance was more pronounced in the *npr1ssn2* double (Figure S1G) and *sni1npr1ssn2* triple (Figure 1D) mutants. To monitor the dynamic change of SSN2 protein levels in response to SA, we generated stable transgenic lines containing the *SSN2-TAP* (tandem affinity purification) fusion construct driven by the native *SSN2* promoter. To confirm the functionality of *SSN2:SSN2-TAP*, we crossed a transgenic line with *sni1ssn2* and the homozygous *sni1ssn2* progeny containing *SSN2:SSN2-TAP* regained the *sni1* phenotype, indicating that the SSN2-TAP fusion protein is biologically active (Figure S1H). The *SSN2:SSN2-TAP* lines were treated with 0.5 mM SA and the SSN2-TAP fusion protein was analyzed at different time points. As shown in Figure 1E, levels of SSN2-TAP protein were upregulated by SA with the highest expression at 8 hr after treatment. This SA-inducible nature of the SSN2 protein, together with the disease phenotype of the *ssn2* mutant, suggests that SSN2 plays a positive role in defense responses.

SSN2 Physically Interacts with SNI1

In view of the genetic interactions between SSN2, RAD51D (SSN1) (Durrant et al., 2007), and SNI1, we explored the possibility of physical interactions between these proteins by using yeast two-hybrid analysis. Because the SNI1 protein exhibited transcriptional repressor activity in yeast (Mosher et al., 2006), both full-length and various truncations of the *SNI1* gene that were made according to previous functional analysis of *SNI1* (Mosher et al., 2006) were cloned into the GAL4 yeast two-hybrid (Y2H) vectors to test interactions. As shown in Figure 2A, strong interaction was revealed between the full-length SNI1 and SSN2. Three SNI1 truncations containing the central region (aa114–233) also interacted with SSN2, suggesting that this part of SNI1 is required for mediating the interaction with SSN2 (Figure 2A). SSN2 contains the SWIM domain, consisting of a $CxCx_nCxH$ motif located downstream of two predicted β strands and followed by an α helix (Makarova et al., 2002). To examine the functional significance of the SWIM domain, we substituted the first conserved cysteine of this motif with a serine residue (C82S). As shown in Figure 2A, the interaction with SNI1 was reduced by the ssn2-C82S mutation. Immunoblot analysis confirmed that similar levels of ssn2-C82S and wild-type SSN2 proteins were produced in these yeast strains (Figure S2A).

Interaction between the N-terminal region of SNI1 and RAD51D was also found, although the full-length SNI1 did not give a positive result (Figure 2B). The C-terminal region of SNI1 has been shown to have transcriptional repressor activity both in yeast and in planta (Mosher et al., 2006), which might have prevented observation of RAD51D interaction with the full-length SNI1 in Y2H. The human RAD51D is known to form a complex with RAD51C (Masson et al., 2001). To determine whether *Arabidopsis* RAD51D associates with RAD51C, we carried out a Y2H assay and detected interaction between these two proteins (Figure 2B). Interestingly, RAD51D was found to also self-interact.

A homolog of SSN2 in fission yeast and in humans, Sws1, partners with RAD51D during homologous DNA recombination (Martín et al., 2006). We examined this aspect of the *Arabidopsis* SSN2 function and confirmed that RAD51D and SSN2 indeed interacted in the Y2H analysis, whereas the interaction with ssn2-C82S was again drastically compromised (Figure 2A). However, SSN2 failed to interact with RAD51C, indicating specificity for its interaction with the RAD51 paralogs (Figure 2A).

To test SNI1 interaction with SSN2 and RAD51D in vivo, we performed coimmunoprecipitation experiments in *Nicotiana benthamiana*. SNI1 was detected in the immunoprecipitates of both SSN2 and RAD51D (Figures 2C and 2D). The specificity of SNI1 interaction with SSN2 was further confirmed by using the GFP protein alone as a negative control (Figure S2B). Moreover, direct interaction between SSN2 and RAD51D was also detected in vivo (Figure 2E) suggesting that SSN2 and RAD51D may function as partners in plants, similar to Sws1 and RAD51D in humans. The discovery of the physical interactions between SSN2, RAD51D, and SNI1 is particularly striking as *ssn2* and *rad51d* are suppressors of *sni1*. This suggests that they may affect each other's activities through physical interactions.

Deficiency in SSN2 Leads to Hypersensitivity to DNA-Damaging Agents

To determine whether the *Arabidopsis* SSN2 has any function in DNA damage repair, we examined the *ssn2* mutant plants for somatic recombination by using an in planta recombination assay with a reporter construct containing two overlapping fragments of the *GUS* gene (Lucht et al., 2002). Recombination events between the two overlapping sequences produce a functional *GUS* gene resulting in blue clusters of cells, which can be visually detected after histochemical staining. The *ssn2* mutant was crossed with the recombination reporter line and the frequency of homologous recombination was measured in the F3 progeny homozygous for the *GUS* reporter in the *ssn2* background. As shown in Figure 3A, the frequency of homologous recombination in the *ssn2* mutant was reduced by half compared to that of the wild-type plant. Furthermore, the *ssn2* mutant also showed a reduced rate of recombination under INA-induced condition, indicating that SSN2, like Sws1 in yeast, plays a role in homologous DNA recombination.

The inability to properly repair DNA damage often leads to increased sensitivity to a variety of DNA-damaging agents (Bray and West, 2005). Mitomycin C (MMC) is an alkylating agent which induces DNA interstrand crosslinking. Plants sensitive to DNA damage agents fail to develop true leaves. Nearly 20% of the *ssn2* mutant plants failed to develop true leaves on MS

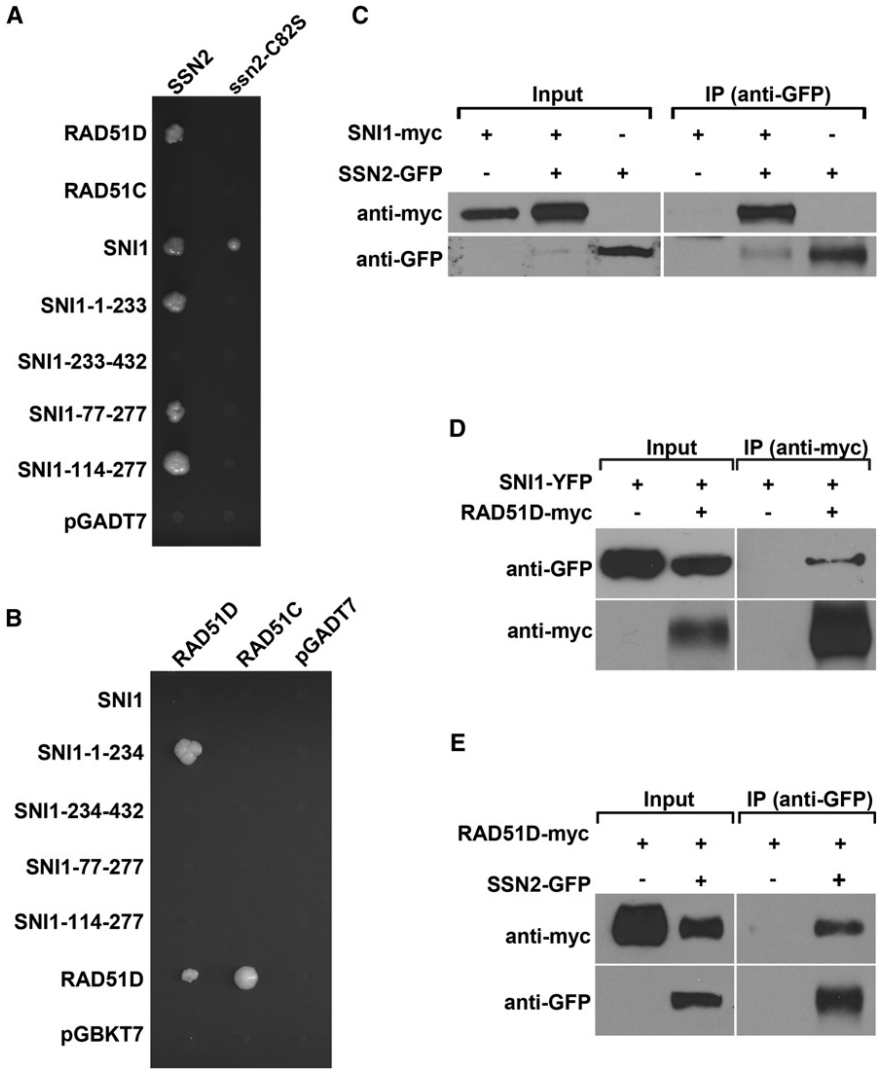

Figure 2. SNI1, RAD51D, and SSN2 Interact with Each Other in Yeast and In Vivo

(A) SSN2 interacts with both SNI1 and RAD51D and the SWIM domain in SSN2 is required for the interaction in yeast. *SSN2* cDNA and ssn2-C82S were cloned into the pGBKT7 vector; *RAD51D*, *RAD51C*, and the full-length *SNI1* and fragments of *SNI1* were cloned into the pGADT7 vector. Interaction was determined by growth on a medium lacking Leu, Trp, His, and Ade. The numbers show the amino acid residues contained in each SNI1 truncation. In the *ssn2-C82S* construct the first cysteine in $CxCx_nCxH$ of the SWIM domain is substituted by a serine.

(B) RAD51D interacts with the N-terminal region of SNI1, RAD51C, and itself in yeast. *SNI1* full-length cDNA, *SNI1* truncations, and *RAD51D* were cloned into the pGBKT7 vector; *RAD51D* and *RAD51C* were cloned into the pGADT7 vector. Yeast two-hybrid assay was performed as in (A).

(C–E) SNI1, SSN2, and RAD51D associate with each other in vivo. All fusion proteins were expressed in *N. benthamiana* plants. Immunoprecipitation was performed by using an anti-GFP antibody (C and E) or an anti-myc agarose conjugate (D) and the immunoblots were analyzed with anti-GFP and anti-myc antibodies. See also Figure S2.

agar medium containing 20 μM MMC, whereas wild-type plants produced normal true leaves at this concentration (Figure 3B). We also tested the sensitivity of the *ssn2* mutant to bleomycin, a γ ray-mimicking agent which can cause double-strand breaks. As expected, the *ssn2* mutant is also hypersensitive to bleomycin in comparison with the wild-type plant (Figure 3B) suggesting an important role of SSN2 in DNA damage repair that is similar to RAD51D (Durrant et al., 2007).

SNI1 and SSN2 Have Inverse Binding Activities to the *PR1* Gene Promoter

The DNA damage repair and defense phenotypes detected in *ssn2* and *rad51d* (Durrant et al., 2007) lead us to ask how the SSN2-RAD51D repair complex affects plant immunity. The compromised basal expression of the *BGL2:GUS* reporter in *sni1ssn2* (Figure 1A) and *sni1rad51d* (Durrant et al., 2007), the delayed expression of the *PR1* gene (Figure 1B) in the *ssn2* single mutant, and the significant blockage in SA induction of defense genes observed in *sni1npr1ssn2* by microarray (Figure 1C) suggest that the SSN2-RAD51D complex may affect defense at the transcriptional level. To determine whether SNI1 and SSN2-RAD51D are directly involved in repressing and activating *PR* gene expression, respectively, we performed a series of chromatin immunoprecipitation (ChIP) experiments by using a set of probes covering both the promoter and the coding regions of *PR1* (Figure 4A). Probes were chosen according to previous promoter studies. Specifically, probe 2 contains a region that when present in the promoter reduced both the basal expression and the inducibility of the gene (Lebel et al., 1998), suggesting that it contains a negative *cis*-acting element. Because this region contains a W-box, it is possible that one of the WRKY transcription factors that are known to negatively regulate *PR* gene expression (Kim et al., 2008; Wang et al., 2006; Xu et al., 2006) functions through this promoter element. Probe 3 contains two *as-1* elements (LS5 and LS7) and one W-box (LS4), which have been shown through linker-scanning mutagenesis to be positive and negative elements, respectively (Lebel et al., 1998). Ample evidence showed that TGA transcription factors regulate *PR1* expression through these *as-1* elements (Johnson et al., 2001; Lam and Lam, 1995; Niggeweg et al., 2000). Probes 1 (−2116 to −1829 bp), 4 (−508 to −262 bp), and 5 (+340 to +429 bp) cover regions not known to affect transcription and therefore serve as negative controls.

To study the SNI1 protein binding, we generated a transgenic line containing the *SNI1-TAP* construct under the control of the native *SNI1* promoter in the *sni1* background. The functionality of this fusion protein was confirmed by restoration of wild-type

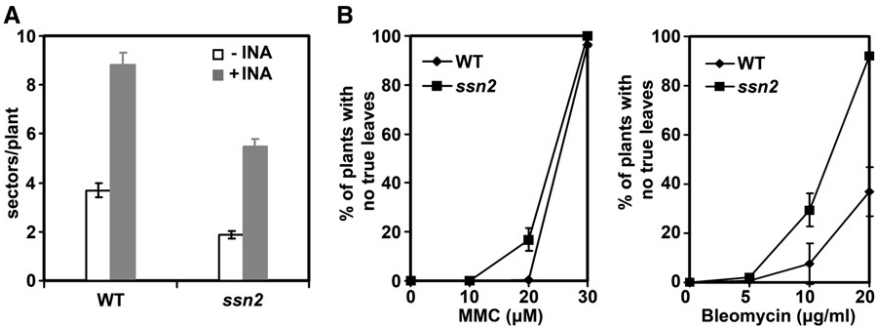

Figure 3. The *ssn2* Mutant Is Defective in Homologous Recombination and Hypersensitive to DNA-Damaging Agents

(A) Frequency of homologous recombination was measured in wild-type (WT) and *ssn2* mutant lines containing the inverted reporter transgene without and with 50 μM INA induction.

(B) The number of plants without true leaves was quantified in WT and *ssn2* mutant plants without and with MMC or bleomycin treatment. Error bars represent SE in both (A) and (B).

morphology (Figure S3A). We first examined SNI1-TAP protein levels in response to 0.5 mM SA treatment and found that they remained constant after SA treatment suggesting that the SNI1 protein is not regulated by changes in protein levels (Figure 4B). ChIP experiments revealed significant enrichments of the probe 2 promoter region comparing the SNI1-TAP sample with the untransformed wild-type plant (WT) (Figure 4C) indicating that SNI1 is associated with the *PR1* promoter in a region that contains a negative *cis*-acting element (e.g., W-box) (Lebel et al., 1998). Moreover, comparing the water-treated SNI1-TAP sample with the SA-treated sample (Figure 4D), we found that the protein binding was reduced upon SA induction. Similar results were obtained by using a transgenic line (*SNI1:SNI1-GFP*) expressing the GFP-tagged SNI1 (Figures S3B and S3C). These data are consistent with SNI1's role as a transcription repressor. Because *sni1* was identified as a suppressor of *npr1*, it is possible that NPR1 activates *PR1* by removing SNI1 from the promoter. To test this hypothesis, we carried out ChIP by using *SNI1:SNI1-TAP* in the *sni1npr1* double mutant. We found that removal of SNI1 from the *PR1* promoter is NPR1 independent (Figure 4E), indicating that a different mechanism is utilized by NPR1 to negate SNI1 repression.

The association of SNI1 with the *PR1* promoter suggests that its suppressor SSN2 may also function at the promoter. To perform ChIP with SSN2, we utilized the *SSN2:SSN2-TAP* lines. As shown in Figure 5A, SSN2 was recruited to the *PR1* promoter in response to SA induction. The binding region defined by ChIP contains probe 2 with the negative element and probe 3 with two of the TGA transcription factor binding *as-1* elements, which are essential for the SA-responsiveness of this gene (Lebel et al., 1998). Because the SSN2 protein level was very low and the enrichment observed was moderate, we performed additional ChIP by using the HA-tagged SSN2 with similar results (Figure S4A).

SSN2 Promoter Binding May Require NPR1 and TGA7

Because NPR1 is not required for SNI1's removal from the promoter, we investigated the possibility that NPR1 is required for the recruitment of SSN2. We first performed ChIP analysis on NPR1 by using the *35S:NPR1-GFP* transgenic lines to determine the promoter region to which NPR1 binds. As shown in Figure 5B, we detected a significant enrichment of the probe 3 region in the SA-treated sample. The NPR1 ChIP experiment was further validated by the finding that NPR1 was also recruited to the promoter of WRKY18, which is another direct target of NPR1 upon induction (Figures S4B and S4C) (Wang et al., 2005). Because both NPR1 and SSN2 bind to the same promoter region, we then investigated whether SSN2 promoter binding requires NPR1. ChIP assay performed in the *npr1* mutant background showed that SSN2 could no longer bind to the *PR1* promoter under induced condition, indicating that the recruitment of SSN2 is dependent on NPR1 (Figure 5C).

This dependency on NPR1 is unlikely to be due to a physical interaction between SSN2 and NPR1, as neither Y2H analysis nor in vivo pull-down experiments showed a positive result. Therefore, we investigated the possibility that SSN2 interacts directly with members of TGA transcription factors, as NPR1 is a known cofactor of TGAs (Després et al., 2000; Zhang et al., 1999; Zhou et al., 2000). In a Y2H analysis, NPR1 interacted with all the TGAs except TGA4 (Tada et al., 2008) and SSN2 showed interaction with only TGA7 (Figure 6A). This interaction was weakened by the ssn2-C82S mutation in the SWIM domain of SSN2. In contrast, RAD51D failed to interact with TGA7 (Figure 6A). Furthermore, the *tga7* mutant showed enhanced susceptibility to *Psm* ES4326 compared to the wild-type plant (Figure 6B), consistent with the idea that TGA7 acts as a transcription activator of *PR* genes. The association between SSN2 and TGA7 was demonstrated in vivo by coimmunoprecipitation when SSN2-myc and TGA7-YFP were transiently expressed in *N. benthamiana* (Figure 6C). TGA7-YFP could pull down SSN2-myc, whereas the negative control, GFP protein alone, failed to do so (Figure S5). To determine whether the SSN2 binding to the *PR1* promoter is dependent on TGA7, we introduced the *SSN2:SSN2-TAP* construct into the *tga7* background by crossing and then carried out ChIP assay in the homozygous progeny. As shown in Figure 6D, no significant enrichment was observed for any of the promoter regions. Therefore, the promoter specificity of SSN2 may be defined through its interaction with the TGA7 transcription factor and depend on the function of the cofactor NPR1.

DISCUSSION

Although genetic data showed that SNI1 negatively regulates defense response, its molecular mechanism has not been clearly defined (Mosher et al., 2006). In this study, we provide evidence that SNI1 suppresses *PR1* gene expression through association with the *PR1* gene promoter region that contains a W-box (Figure 7). Because no discernible DNA binding domain has been identified in the SNI1 protein, the binding may be indirect and perhaps mediated by a WRKY transcription factor that plays a negative role in defense signaling. WRKY18, WRKY40, and WRKY60 have been shown to function redundantly and

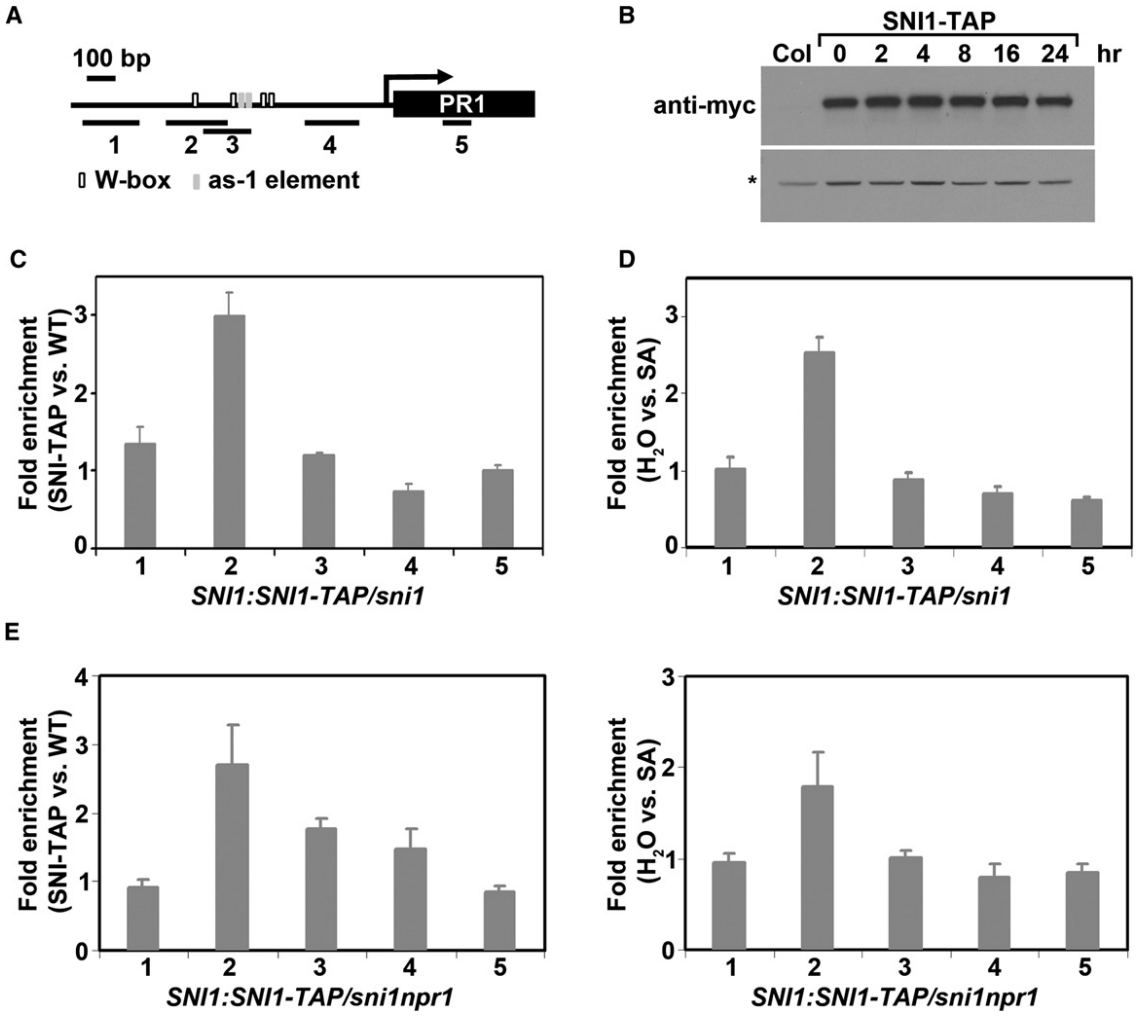

Figure 4. SNI1 Binds to the *PR1* Gene Promoter and Is Removed from the Promoter in an SA-Dependent and NPR1-Independent Manner

(A) Schematic drawing of the *PR1* locus, *cis*-acting elements, and probe locations for ChIP assay.

(B) The SNI1 protein level is constant with and without SA treatment. Three-week-old *SNI1:SNI1-TAP* plants were treated with 0.5 mM SA. Proteins were extracted at the indicated times and detected with an anti-myc antibody.

(C) SNI1 constitutively binds to the *PR1* promoter. ChIP analysis was performed by using the *sni1* plants expressing *SNI1:SNI1-TAP* (*SNI1:SNI1-TAP/sni1*). ChIP data from the untransformed plants (WT) were used as the control.

(D) SNI1 is removed from the *PR1* gene promoter upon induction. ChIP analysis was performed on *SNI1:SNI1-TAP/sni1* plants treated with water or 0.5 mM SA for 16 hr.

(E) Removal of SNI1 from the *PR1* gene promoter is NPR1 independent. ChIP analysis was performed as in (C) and (D) but in the *sni1npr1* background (*SNI1:SNI1-TAP/sni1npr1*). Values are mean ± SE from three technical replicates of one representative experiment. All ChIP assays were performed at least three times with similar results. See also Figure S3.

cooperatively to suppress the expression of some defense-related genes (Xu et al., 2006). Moreover, WRKY38 and WRKY62 were found to negatively regulate plant-defense response through interaction with histone deacetylase 19 (Kim et al., 2008). Whether and which WRKY transcription factor associates with SNI1 will be the subject of further investigation.

The repressor function of SNI1 cannot be explained solely by its occupancy of the promoter region to which positive regulators such as SSN2 bind because in the *npr1* mutant SNI1 can still be removed from the promoter upon induction while no *PR1* is transcribed. Therefore, we hypothesize that SNI1 binding to the promoter may bring about a chromatin structure that is unfavorable for transcription, perhaps through recruitment of a chromatin-remodeling protein such as a histone deacetylase (shown as X in Figure 7). This closed chromatin structure is reversed by NPR1, perhaps partly through the activity of SSN2.

The physical interaction between SSN2 and TGA7 both in yeast and in planta suggests that SSN2 is recruited to the *PR* gene promoter through TGA7, which is known to bind to the same promoter elements in electrophoretic mobility shift assay (Shearer et al., 2009). Interestingly, of the TGA transcription factors, TGA7 is the only one that interacts with SSN2 in Y2H analysis. This may explain the significant disease susceptibility phenotype observed in the *tga7* single mutant. However, *tga7* is not as susceptible as *npr1* because of the presence of other TGAs, which are able to induce *PR1* expression in the presence

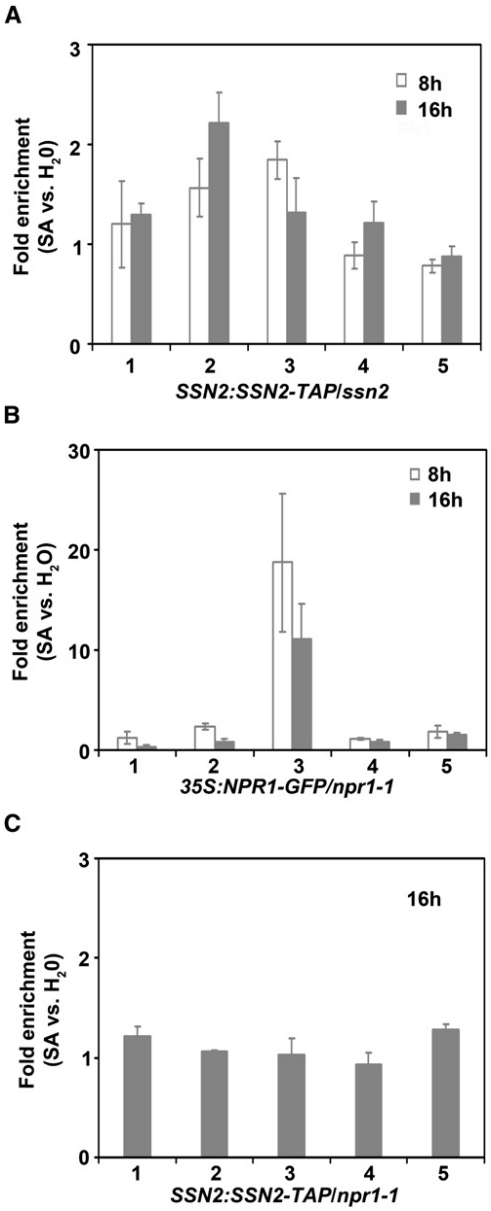

Figure 5. SSN2 Is Recruited to the *PR1* Gene Promoter in an SA- and NPR1-Dependent Manner

(A) Recruitment of SSN2 is dependent on SA. ChIP analysis on plants expressing *SSN2:SSN2-TAP* in the *ssn2* background (*SSN2:SSN2-TAP/ssn2*) treated with water or 0.5 mM SA for 8 or 16 hr.

(B) Recruitment of NPR1 is dependent on SA. ChIP assay on plants expressing *35S:NPR1-GFP* treated with water or 0.5 mM SA for 8 or 16 hr.

(C) Recruitment of SSN2 requires NPR1. ChIP assay on plants expressing *SSN2:SSN2-TAP* in the *npr1-1* background (*SSN2:SSN2-TAP/npr1-1*) treated with water or 0.5 mM SA for 16 hr. Values are mean ± SE from three technical replicates of one representative experiment. All ChIP assays were performed at least three times with similar results. See also Figure S4.

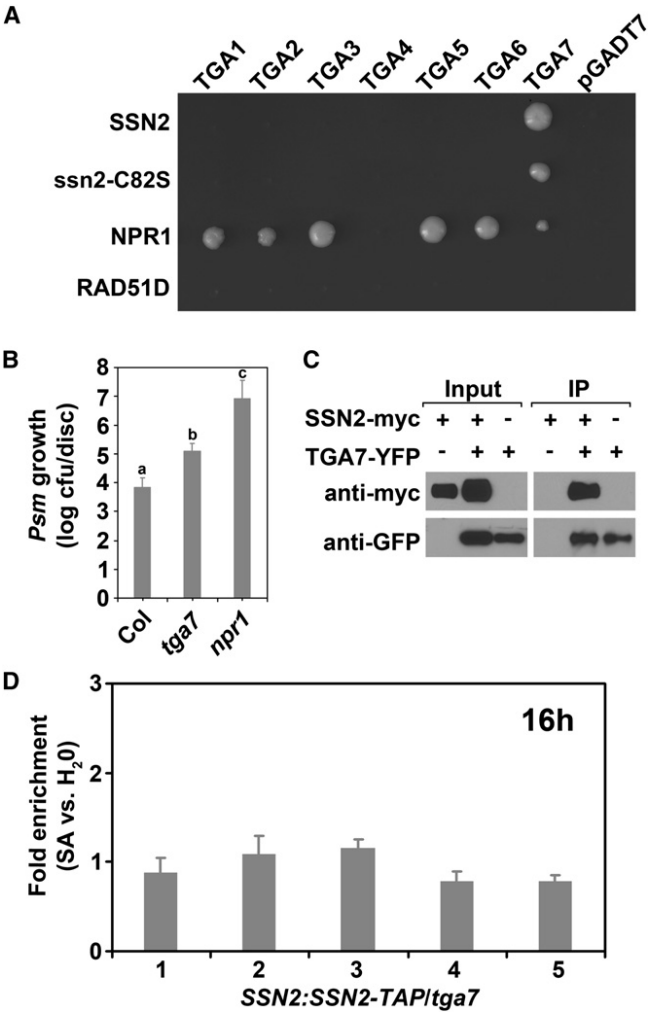

Figure 6. SSN2 Interacts with TGA7

(A) SSN2 interacts with TGA7 in yeast. *SSN2*, ssn2-C82S, *NPR1*, and *RAD51D* were cloned into the pGBKT7 vector; *TGA*s were cloned into the pGADT7 vector. Interaction was determined by growth on a medium lacking Leu, Trp, His, and Ade.

(B) TGA7 is a positive regulator of defense. Three-week-old soil-grown plants were infiltrated with *Psm* ES4326 (OD_{600} = 0.0001) and colony-forming-units (cfu) were quantified 3 days after inoculation. Error bars represent 95% confidence limits. Different letters indicate statistically significant differences between genotypes ($p < 0.01$). The experiments were repeated three times with similar results.

(C) SSN2 interacts with TGA7 in vivo. SSN2-myc and TGA7-YFP fusion proteins were expressed in *N. benthamiana* plants. Immunoprecipitation was performed by using an anti-GFP antibody and the immunoblot was analyzed with anti-GFP and anti-myc antibodies.

(D) Recruitment of SSN2 is dependent on TGA7. ChIP assay on plants expressing *SSN2:SSN2-TAP* in the *tga7* background (*SSN2:SSN2-TAP/tga7*) treated with water or 0.5 mM SA for 16 hr. Values are mean ± SE from three technical replicates of one representative experiment. This experiment was repeated twice with similar results. See also Figure S5.

of NPR1. Moreover, SA-induced recruitment of SSN2 is NPR1 dependent. Therefore, either activation of TGA by NPR1 or NPR1-dependent SNI1 inactivation and SSN2 binding at the promoter region is sufficient to cause *PR1* gene induction. In the absence of SNI1, the chromatin is more accessible and NPR1 is no longer required for SSN2 binding and *PR1* gene transcription. This is consistent with previous promoter linker-scanning studies demonstrating that the *PR1* promoter contains multiple positive and negative *cis*-acting elements (Lebel et al., 1998).

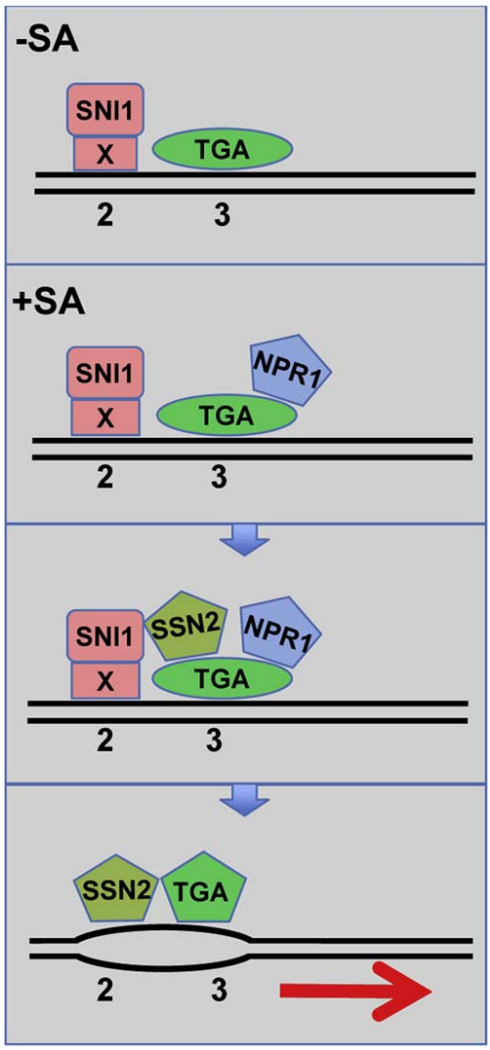

Figure 7. Working Model for the Regulation of *PR1* Gene Promoter by SNI1, SSN2, and NPR1

In the absence of induction, SNI1 is associated with the *PR1* gene promoter. Together with a histone modification protein (X), such as a histone deacetylase, SNI1 keeps the chromatin in a closed state to prevent gene expression. In response to induction (+SA), with the help of NPR1, SSN2 is recruited to the *PR1* gene promoter to reverse SNI1-generated repression. Consequently *PR1* is induced as a result of NPR1-mediated activation of TGA transcription factors and binding of SSN2.

This model is also further supported by our current knowledge of SSN2 through studies in other organisms. In fission yeast, the SSN2 homolog Sws1 can interact with DNA helicase Srs2, whose C-terminal noncatalytic domain mediates its interaction with RAD51 (Krejci et al., 2003; Martín et al., 2006). RAD51D was also recently found to form a complex with RAD51 (Tambini et al., 2010) that partners with RAD54, a member of the SWI2/SNF2 family of ATPases. RAD51 and RAD54 function cooperatively in the ATP-dependent remodeling of chromatin (Alexeev et al., 2003; Alexiadis and Kadonaga, 2002). Moreover, we recently found that RAD51 can also bind to the *PR* gene promoters in *Arabidopsis* upon SA induction (Wang et al., 2010). These discoveries lead us to hypothesize that the SSN2-RAD51D complex may activate defense gene expression by opening chromatin via the action of a protein like RAD54.

The homologous recombination pathway has been well-characterized in yeast and mammalian systems. We have uncovered a previously unknown function of these DNA repair proteins in direct regulation of gene transcription in response to infection. Our data illustrate that complex mechanisms are involved in coordinating the actions of negative regulator SNI1, positive regulator SSNs, and coactivator NPR1 to achieve tight control on plant immune genes. Because the components of SSN2-RAD51D complex, as well as their interaction, are conserved in eukaryotes from fission yeast to humans and plants, our discovery of their role in transcription may have broad implications in general biology.

EXPERIMENTAL PROCEDURES

Gene Expression Analysis

Total RNA was extracted from 3-week-old soil-grown plants treated with 0.5 mM SA at different time points. First-strand cDNA was synthesized by using the Superscript®III reverse transcriptase (Invitrogen). Quantitative PCR was carried out on cDNA diluted 20 times by using QuantiTect SYBR Green PCR Kit (QIAGEN) in a LightCycler (Roche) according to the manufacturer's instructions. Gene expression analysis was performed as previously described with cDNA samples normalized by using *ubiquitin5* (Kesarwani et al., 2007). The primer sequences used for qPCR are listed in Table S1.

Microarray Analysis

Total RNA was extracted from 3-week-old plants 16 hr after treatment with water or 0.5 mM SA. The RNA labeled with MessageAmp Premier RNA Amplification Kit (Ambion) was hybridized with GeneChip *Arabidopsis* ATH1 Genome Array (Affymetrix) and subsequently washed and scanned at the Duke Microarray Facility. Experiments were carried out in triplicate. The microarray data were normalized by using Gene-Spring GX Software (RMA algorithm; Agilent). The two-way ANOVA with Benjamini-Hochberg multiple comparison correction was used to identify SSN2-dependent genes (significant interaction between genotypes and treatments, $p < 0.01$). The SA-induced genes were found through unpaired Student's t test with Benjamini-Hochberg multiple comparison correction. The gene ontology analysis was performed by using the corresponding module in Gene-Spring.

Bacterial Infection Assay

Infection of *Arabidopsis* plants with *Pseudomonas syringae* pv *maculicola* ES4326 was performed as described previously (Durrant et al., 2007).

Yeast Two-Hybrid Analysis

Yeast two-hybrid analysis was performed by using the Matchmaker Gal4 system following the manufacturer's instructions (Clontech). Bait cDNAs were cloned into the pGBKT7 vector and transformed into yeast strain Y187 (*MAT*α). Prey cDNAs were cloned into the pGADT7 vector and transformed into yeast strain AH109 of the opposite mating type (*MATa*). Protein-protein interaction was determined by growth rate of mating zygotes on SD-Leu-Trp-His-Ade plates.

Coimmunoprecipitation

The cDNAs of *SSN2*, *SNI1*, *RAD51D*, and *TGA7* were cloned into the pMDC83 (GFP), pGWB20 (10 × myc), or pEG101 (YFP) vectors to generate *SSN2-GFP*, *SNI1-myc*, *RAD51D-myc*, *SNI1-YFP*, *TGA7-YFP*, and *SSN2-myc* constructs, which were transformed into *Agrobacterium tumefaciens* strain GV3101. For transient protein expression in *N. benthamiana*, *Agrobacterium* cultures were infiltrated into 4-week-old plants. Leaves were collected and processed 3 days later. For the coimmunoprecipitation experiment with SSN2 and RAD51D, the leaf tissues were crosslinked with 2 mM dithiobis (succinimidyl propionate) (DSP; Pierce) for 30 min at room temperature to stabilize protein-protein interaction. The crosslinking reaction was quenched by

addition of 1M Tris to a final concentration of 50 mM. Protein extraction was carried out by homogenizing leaf tissue in extraction buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM EDTA, 0.1% Triton X-100, 10% glycerol, 40 μM MG115, and protease inhibitor cocktail at 1:100 [Sigma-Aldrich]). Homogenates were centrifuged three times at 16,000 *g* for 10 min at 4°C. Immunoprecipitation was performed by using the anti-myc agarose (Sigma-Aldrich) or the anti-GFP antibody (Abcam) followed by incubation with Dynabeads protein G (Invitrogen). The precipitated protein eluted with the SDS loading buffer and 1% of the total protein used as input were subjected to SDS-PAGE and immunoblotted with various antibodies.

In Planta Recombination Assay

The recombination reporter line 1445 containing overlapping segments of the *GUS* gene in inverted orientation (Lucht et al., 2002) was crossed into the *sni1ssn2* mutant carrying the *BGL2:GUS* transgene. A homozygous line containing the recombination reporter gene in the *ssn2* single mutant background was isolated and recombination frequencies were measured as previously described (Durrant et al., 2007).

Mitomycin C and Bleomycin Sensitivity Assay

Plants were grown on MS media containing different concentrations of mitomycin C (0, 10, 20, or 30 μM) or bleomycin (0, 5, 10, or 20 μg/ml) (Sigma-Aldrich) for 14 days and scored for production of true leaves. Sensitivity was indicated by the percentage of plants without true leaves.

Chromatin Immunoprecipitation

ChIP assay was performed as described previously (Gendrel et al., 2005). Briefly, 1 g of 9- to 12-day-old *Arabidopsis* seedlings grown on MS plates treated with water or 0.5 mM SA for 8 or 16 hr was crosslinked with 1% formaldehyde under vacuum for 15 min at room temperature. The crosslinking reaction was stopped by adding glycine to a final concentration of 0.125 M. The seedlings were washed with water and then ground in liquid nitrogen. *Arabidopsis* nuclei were isolated and sonicated to shear DNA into 500 to 1000 bp fragments by using Branson Digital Sonifier 250 (Branson Ultrasonics) followed by immunoprecipitation. For the SSN2-TAP and SNI1-TAP proteins, IgG Sepharose 6 Fast Flow beads (GE Healthcare Biosciences) were used. For the GFP-tagged proteins, anti-GFP (Abcam) was first coupled to protein G Dynabeads (Invitrogen) and then used for immunoprecipitation. The purified ChIP samples were subject to qPCR analysis. To normalize the results, we quantified the amount of *18S rRNA* gene sequence as the internal control. Fold enrichment was determined by the relative ratio of the normalized ChIP signals between samples. The primer sequences used for ChIP are listed in Table S1.

ACCESSION NUMBERS

The microarray data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database (accession number GSE23617).

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures, one table, Supplemental Experimental Procedures, and one data file and can be found with this article online at doi:10.1016/j.chom.2011.01.011.

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