The Arabidopsis *PR-1* Promoter Contains Multiple Integration Sites for the Coactivator NPR1 and the Repressor SNI1[W]

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Systemic acquired resistance is a broad-spectrum plant immune response involving massive transcriptional reprogramming. The Arabidopsis (*Arabidopsis thaliana*) *PATHOGENESIS-RELATED-1* (*PR-1*) gene has been used in numerous studies to elucidate transcriptional control mechanisms regulating systemic acquired resistance. WRKY transcription factors and basic leucine zipper proteins of the TGA family regulate the *PR-1* promoter by binding to specific cis-elements. In addition, the promoter is under the control of two proteins that do not directly contact the DNA: the positive regulator NONEXPRESSOR OF *PR* GENES1 (NPR1), which physically interacts with TGA factors, and the repressor SUPPRESSOR OF NPR1, INDUCIBLE1 (SNI1). In this study, we analyzed the importance of the TGA-binding sites *LS5* and *LS7* and the *WKRY* box *LS4* for regulation by NPR1 and SNI1. In the absence of *LS5* and *LS7*, NPR1 activates the *PR-1* promoter through a mechanism that requires *LS4*. Since transcriptional activation of *WRKY* genes is under the control of NPR1 and since *LS4* is not sufficient for the activation of a truncated *PR-1* promoter by the effector protein NPR1-VP16 in transient assays, it is concluded that the *LS4*-dependent activation of the *PR-1* promoter is indirect. In the case of NPR1 acting directly through TGA factors at its target promoters, two TGA-binding sites are necessary but not sufficient for NPR1 function in transgenic plants and in the NPR-VP16-based transactivation assay in protoplasts. SNI1 exerts its negative effect in the noninduced state by targeting unknown proteins associated with sequences between bp −816 and −573. Under induced conditions, SNI1 negatively regulates the function of WRKY transcription factors binding to *WKRY* boxes between bp −550 and −510.

The plant signaling hormone salicylic acid (SA) is synthesized upon pathogen infection and serves to elicit plant defense reactions (Malamy et al., 1990; Vlot et al., 2009). Increased SA levels in systemic leaves lead to the onset of systemic acquired resistance (SAR), an inducible defense program that renders the plant immune against a broad spectrum of pathogens (Ross, 1961; Durrant and Dong, 2004). Treatment of plants with SA or its analogs 2,6-dichloro-isonicotinic acid (INA) or benzothiadiazole *S*-methyl ester is sufficient for SAR establishment.

The signaling cascade leading to the activation of SAR has been elaborated in Arabidopsis (*Arabidopsis thaliana*) using SA-inducible *PATHOGENESIS-RELATED* (*PR*) genes (Cao et al., 1994; Lebel et al., 1998; Zhang et al., 2003; Rochon et al., 2006; Kesarwani et al., 2007). After performing a mutant screen designed to isolate plants that cannot activate a *PR-2*:*GUS* reporter construct in the presence of SA or INA, Cao and colleagues (Cao et al., 1994, 1997; Wang et al., 2006) identified the protein NONEXPRESSOR OF *PR* GENES1 (NPR1), which is essential for the regulation of 2,248 genes after benzothiadiazole *S*-methyl ester treatment and for SAR establishment. In the absence of SA, the majority of the NPR1 proteins reside in the cytosol as an oligomer (Mou et al., 2003). Upon pathogen attack or INA treatment, NPR1 becomes reduced and the oligomer dissociates. NPR1 monomers are translocated into the nucleus, where they interact with TGA factors (Zhang et al., 1999). TGA factors constitute a subfamily of basic Leu zipper transcription factors that recognize TGACG motifs found in NPR1-dependent (Zhang et al., 1999; Thibaud-Nissen et al., 2006) but also in NPR1-independent (Fode et al., 2008; Blanco et al., 2009) SA-inducible promoters. Mutation of the three redundant members of clade II TGA factors, TGA2, TGA5, and TGA6, leads to increased basal *PR-1* expression levels that cannot be induced any longer (Zhang et al., 2003).

The negative regulatory protein SUPPRESSOR OF NPR1, INDUCIBLE1 (SNI1) was identified in a genetic screen for mutants that induce the *PR-2*:*GUS* reporter construct in the *npr1* mutant background (Li et al., 1999). In *sni1 npr1* plants, INA induction of the *PR-1* promoter is reestablished, unraveling an NPR1-independent pathway that can activate the promoter in the absence of SNI. This observation implies that NPR1 is required to inactivate SNI1. The molecular mechanism that drives the NPR1/SNI1-independent induction pathway is not yet understood, but it requires the recombination/repair

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protein RAD51D (Durrant et al., 2007). In the presence of NPR1, SNI1 dampens both basal and induced expression of *PR* genes. Chromatin immunoprecipitation experiments indicate that histone modifications are involved in SNI1-mediated repression (Mosher et al., 2006).

Deletion and linker-scanning (LS) mutations have identified functionally important cis-regulatory sequences between positions −698 and −621 of the *PR-1* promoter (Lebel et al., 1998). Interestingly, two 10-bp LS substitutions that affect the TGA-binding sites *LS5* and *LS7* (Fig. 1A) had different effects on promoter activity: substitution of *LS7* by a linker sequence led to a complete loss of promoter activity, whereas mutation of *LS5* led to enhanced basal expression levels, which could be induced to wild-type-like levels by INA. Thus, it appeared that the two TGA-binding sites have different functions. Based on the finding that TGA factors physically interact with the positive regulator NPR1 (Zhang et al., 1999; Fan and Dong, 2002), it was suggested that *LS7* facilitates NPR1-mediated activation of the promoter. As TGA factors repress the promoter in the uninduced state (Zhang et al., 2003; Boyle et al., 2009), it was proposed that their binding to *LS5* mediates the repression of basal levels (Kesarwani et al., 2007). Mutation of *LS4*, which encodes a potential target site for WRKY transcription factors, enhanced promoter activity (Lebel et al., 1998). Therefore, this site was postulated to be involved in the repressive effect of SNI1 (Kesarwani et al., 2007; for a

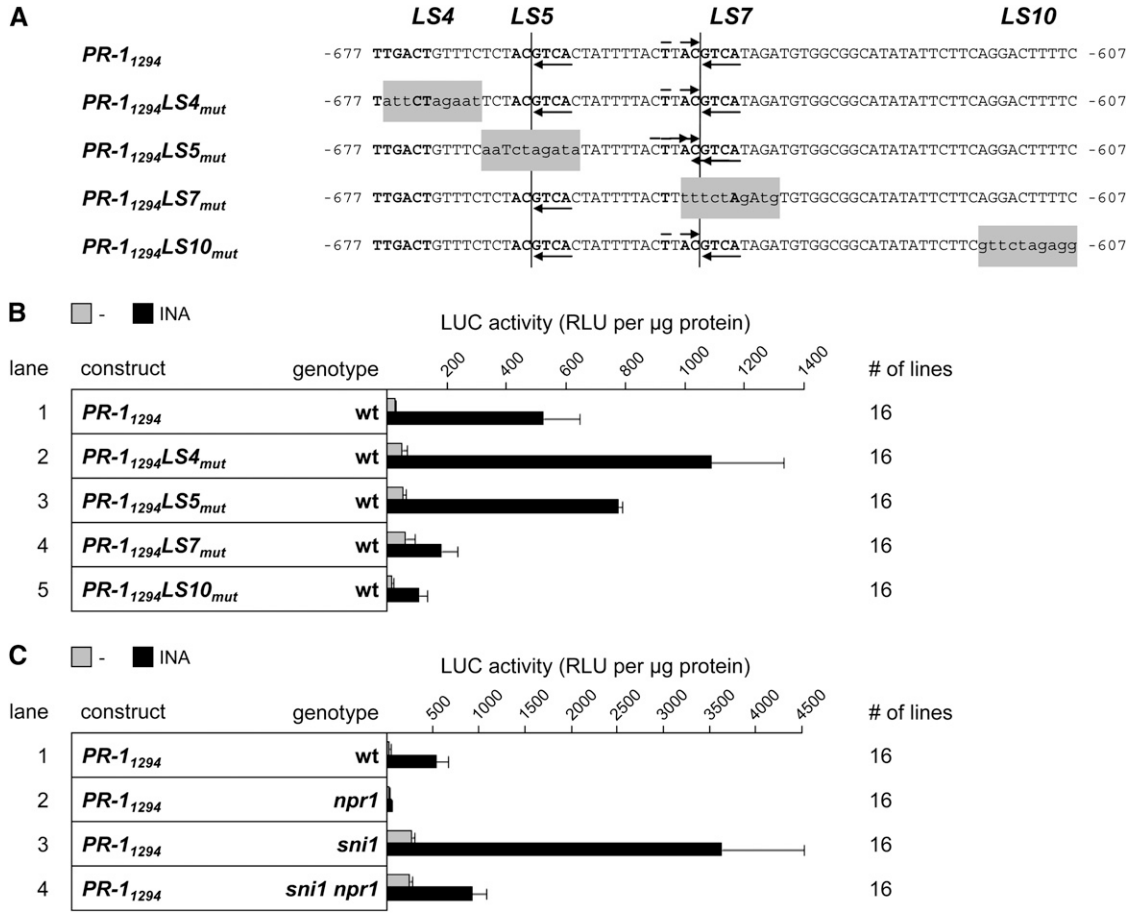

Figure 1. Influence of the cis-elements *LS4*, *LS5*, *LS7*, and *LS10* and the trans-factors NPR1 and SNI1 on *PR-1*$_{1294}$ promoter activity. A, Mutations introduced into the *PR-1*$_{1294}$ promoter context to study the functional significance of *LS4*, *LS5*, *LS7*, and *LS10*. The *W* box of *LS4* and conserved positions within the ideal TGA-binding site TGAC/GTCA are depicted in boldface letters; each TGAC half-site is marked by an arrow provided that 3 or 4 bp are in consensus with this motif. Vertical lines denote the centers of the palindromes. The LS mutations as originally introduced by Lebel et al. (1998) are shown in gray boxes and lowercase letters. B, Influence of *LS4*, *LS5*, *LS7*, and *LS10* on *PR-1*$_{1294}$ promoter activity. C, Expression of *PR-1*$_{1294}$ in *npr1*, *sni1*, and *sni1 npr1*. For B and C, LUC activities of 2-week-old plants grown axenically on MS plates without (gray columns) and with (black columns) 30 μM INA were measured. The indicated *LS* elements were replaced by linker sequences within the *PR-1*$_{1294}$ promoter context. The genotypes of the analyzed plants are indicated. Values represent means of the activities of the indicated number (#) of independent lines. LUC activities are expressed as relative light units (RLU) per μg of total protein. Error bars represent the SD of two independent experiments. wt, Wild type.

schematic representation of the current model, see Supplemental Fig. S1). *LS10*, a potential binding site for DOF (DNA binding with one finger) transcription factors, is, like *LS7*, of crucial importance for the activation of the promoter.

Since NPR1, SNI1, and TGA transcription factors are key regulators of SAR, the elucidation of their mode of action is of major interest. Recent studies have yielded further results (Mosher et al., 2006; Rochon et al., 2006; Zhang et al., 2006; Durrant et al., 2007; Tada et al., 2008; Boyle et al., 2009; Spoel et al., 2009) that need to be integrated into the current model of their interplay on the *PR-1* promoter (Kesarwani et al., 2007). Since this model is based on two different studies, expression analysis of the endogenous promoter in different genetic backgrounds (Kesarwani et al., 2007) and deletion and LS analysis of different *PR-1* promoter variants in transgenic wild-type plants (Lebel et al., 1998), we challenged the postulated significance of *LS4*, *LS5*, and *LS7* for the functions of NPR1, SNI1, and TGA factors in one experimental setup: we generated numerous *PR-1* promoter mutants and compared their expression levels in the *npr1*, *sni1*, and *sni1 npr1* genetic backgrounds. Unexpectedly, we found that simultaneous mutation of *LS5* and *LS7* did not disrupt NPR1-dependent expression of the *PR-1* promoter. This *LS5/LS7*-independent promoter activity is repressed by *LS5* and appears to be driven by *LS4*-bound WRKY transcription factors whose expression is regulated by SA and NPR1. In the case of NPR1 acting through TGA-binding sites at target promoters, two TGA-binding motifs as well as not yet identified sequences between these motifs are required for NPR1 function. Likewise, efficient repression of the promoter by TGA factors is mediated by an intact *LS5/LS7* motif. In contrast to previous assumptions (Kesarwani et al., 2007), SNI1 acts independently from *LS4*. In the noninduced state, it targets yet unknown sequences between −816 and −573. Further *WKRY* boxes (*W* boxes) outside this region are required for hyperinduction in *sni1*. An updated model of the transcriptional control mechanisms operating on the *PR-1* promoter is presented.

RESULTS

The 1,294-bp-Long *PR-1* Promoter Fragment Is Regulated Like the Endogenous Gene in *npr1*, *sni1*, and *sni1 npr1*

As treatment of plants with the SA analog INA is sufficient for SAR establishment and *PR-1* induction, *PR-1* transcription was monitored either 3 d after spraying Murashige and Skoog (MS) plate-grown plantlets with 350 μM INA (Lebel et al., 1998) or after growing seedlings for 14 d on 30 μM INA (Kesarwani et al., 2007). Since the latter treatment had been successfully deployed for the isolation of the two SAR-regulating proteins, NPR1 (Cao et al., 1994) and SNI1 (Li et al., 1999), we decided to base our analysis on this induction procedure. As displayed in Figure 1B, this treatment basically recapitulated the results reported before (Lebel et al., 1998): induction of a *luciferase* (*LUC*) reporter gene construct under the control of a 1,294-bp-long *PR-1* promoter ($PR\text{-}1_{1294}$) was enhanced when substituting *LS4* by a linker sequence; replacement of only *LS5* did not interfere with INA induction, and substitution of either *LS7* or *LS10* compromised promoter activity. Quantitative differences from the previous report (Lebel et al., 1998), like the less pronounced enhanced basal expression levels of $PR\text{-}1_{1294}LS4_{mut}$ and $PR\text{-}1_{1294}LS5_{mut}$, might be the result of the different reporter gene used in this study. Because of the high stability of the GUS enzyme used by Lebel et al. (1998), background levels might have accumulated to a higher degree, thus exaggerating subtle effects on basal expression levels.

When testing the $PR\text{-}1_{1294}$:*LUC* construct in *npr1*, *sni1*, and *sni1 npr1* plants, we observed that it displayed the relevant characteristics of the endogenous *PR-1* promoter (Fig. 1C; Li et al., 1999). Induction was severely compromised in the *npr1* mutant, whereas basal and induced levels were enhanced in the *sni1* mutant, demonstrating that the sequences necessary for the regulation of *PR-1* by NPR1 and SNI1 are located within 1,294 bp upstream of the transcriptional start site. The described NPR1/SNI1-independent activation mode was also functional (Fig. 1C, lane 4), although with a higher efficiency than described before for the endogenous gene: LUC activities reached the same induced values in wild-type plants as in *sni1 npr1* plants, whereas the endogenous *PR-1* RNA levels were less abundant in *sni1 npr1* (Li et al., 1999; Supplemental Fig. S2). It might well be that the NPR1/SNI1-independent activation mode is negatively affected by sequences outside the 1,294-bp promoter fragment used in this study.

NPR1-Dependent INA-Induced Activation of the *PR-1* Promoter Occurs Even in the Absence of TGA-Binding Sites

Since *LS5* and *LS7* are both recognized by TGA transcription factors, it had remained elusive why only mutation of *LS7* interfered with INA-inducible expression. Unexpectedly, simultaneous mutation of *LS5* and *LS7* within $PR\text{-}1_{1294}$ yielded a promoter that responded to INA almost like the wild-type promoter (Fig. 2, lane 2), although expression levels were somehow higher both in the noninduced state and the induced state. Thus, mutating *LS5* suppresses the negative effect of the *LS7* mutation.

In order to address the question of whether removal of the TGA-binding sites affects the influence of the TGA-interacting protein NPR1 and its suppressor SNI1, we analyzed the expression of the $PR\text{-}1_{1294}LS5,7_{mut}$ promoter in *npr1* and *sni1* mutant plants. Although the putative NPR1 recruitment site *LS7* was mutated, NPR1 was still required for the activation (Fig. 2, lane 3). Thus, an NPR1-dependent activation mode that does not depend on the TGA-binding sites must exist.

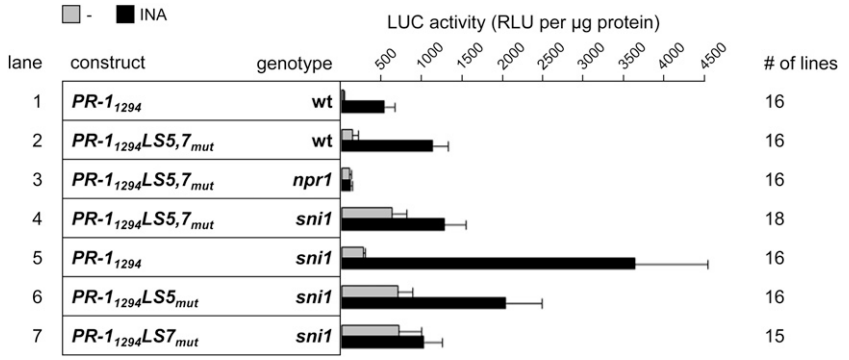

Figure 2. Influence of the simultaneous mutation of the TGA-binding motifs *LS5* and *LS7* on *PR-1*$_{1294}$ promoter activity. LUC activities of 2-week-old plants grown axenically on MS plates without (gray columns) and with (black columns) 30 μM INA were measured. The indicated *LS* elements were replaced by linker sequences within the *PR-1*$_{1294}$ promoter context (Fig. 1A). The genotypes of the analyzed plants are indicated. Values are means of the activities of the indicated number (#) of independent lines. LUC activities are expressed as relative light units (RLU) per μg of total protein. Error bars represent the SD of two independent experiments. wt, Wild type.

In the *sni1* mutant, basal expression levels of the *PR-1*$_{1294}$*LS5,7*$_{mut}$ promoter were higher than those of the wild-type promoter (Fig. 2, compare lanes 4 and 5) and were comparable to those of the induced wild-type promoter in the wild-type background (Fig. 2, compare lanes 1 and 4). This indicates that basal levels of the *PR-1* promoter are suppressed by two independent mechanisms: by TGA factors at *LS5/LS7* as well as by SNI1. In the presence of INA, the *sni1* allele had no effect on expression of *PR-1*$_{1294}$*LS5,7*$_{mut}$ (Fig. 2, compare lanes 2 and 4).

LS substitutions in only one of the two TGA-binding sites, as in *PR-1*$_{1294}$*LS5*$_{mut}$ and *PR-1*$_{1294}$*LS7*$_{mut}$, yielded high basal expression levels in *sni1*, indicating that one binding site is not sufficient for efficient repression (Fig. 2, lanes 6 and 7). In contrast, hyperinduction of *PR-1*$_{1294}$ in *sni1* was severely compromised when mutating *LS7* and to a lesser degree when mutating *LS5* (Fig. 2, compare lane 5 with lanes 6 and 7). It is concluded that *LS5* and *LS7* are both needed for efficient hyperactivation in *sni1*.

The *LS5*/*LS7*-Independent Pathway Requires the *W* box *LS4*

The NPR1 dependency of the *PR-1*$_{1294}$*LS5,7*$_{mut}$ promoter (Fig. 2, lane 3) implied that it might be indirectly regulated through *WRKY* genes that are directly upregulated by NPR1 (Wang et al., 2006). Although *LS4* (a *W* box located upstream of *LS5*) dampens promoter activity (Lebel et al., 1998; Fig. 1B, lane 2), we considered this motif as a candidate sequence to be responsible for the activation of *PR-1* lacking the *LS5/LS7* motif. Indeed, induction of a promoter carrying linker substitutions of *LS4*, *LS5*, and *LS7* (*PR-1*$_{1294}$*LS4,5,7*$_{mut}$) was severely compromised (Fig. 3, lane 3). It is concluded that *LS4* is required for the activation of the promoter in the absence of *LS5* and *LS7*. In the presence of these two motifs, *LS4* is dispensable for induction (Fig. 3, lane 4). The promoter constructs *PR-1*$_{1294}$*LS4,5*$_{mut}$ and *PR-1*$_{1294}$*LS4,7*$_{mut}$ did not show any activities, indicating that neither *LS5* nor *LS7* is sufficient for activation of the promoter in the absence of *LS4* (Fig. 3, lanes 5 and 6).

The *LS4*-Independent Pathway Requires Two TGA-Binding Sites

The results displayed in Figure 3 suggest that two alternative activation pathways exist: one that is driven by *LS4*, and a second that requires *LS5* and *LS7*. Since chromatin immunoprecipitation analysis has demonstrated an association of NPR1 with the *PR-1* promoter (Rochon et al., 2006) and since NPR1 directly interacts with TGA factors (Zhang et al., 1999), we assume that the second pathway works through the direct association of NPR1 with TGA factors bound to the *LS5/LS7* motif. As we were unable to obtain a suitable anti-NPR1 antiserum for chromatin immunoprecipitation assays, we developed a transient assay that would support our idea that activation through *LS4* is not mediated through a mechanism that recruits NPR1 whereas the combined action of *LS5* and *LS7* would be able to do so. To this end, truncated *PR-1* promoter derivatives were constructed that contain the sequences from −816 to −573, including *LS4*, *LS5*, and *LS7* upstream of the *PR-1* core promoter (−68 to +1; Fig. 4A). When fused to the *LUC* gene, this *PR-1*$_{816–573}$ promoter yields low background activity when transfected into SA-treated *npr1* protoplasts (Fig. 4B). Coexpression of NPR1 or NPR1 fused to the activation domain of herpes simplex virus protein 16 (VP16) led to a 5- and 20-fold enhancement of promoter activity, respectively (Supplemental Fig. S3). Because of the higher activation capacity of NPR1-VP16, subsequent studies were performed with this construct. This activation was enhanced by SA (Supplemental Fig. S4) and was abolished after mutation of *LS5* and *LS7* (Fig.

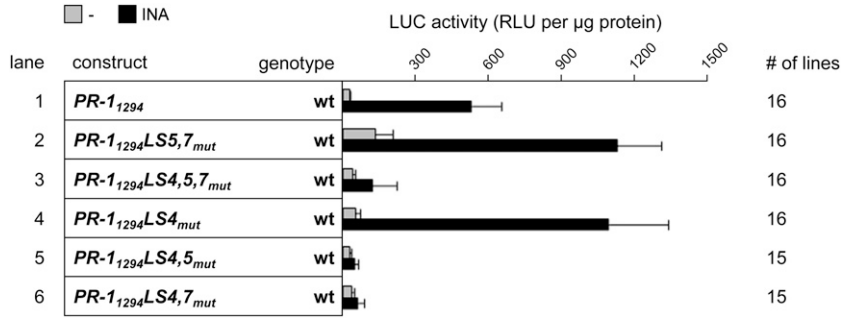

Figure 3. Influence of *LS4* on *PR-1*$_{1294}$ promoter activity. LUC activities of 18-d-old plants grown axenically on MS plates without (gray columns) and with (black columns) 30 μM INA were measured. The indicated *LS* elements were exchanged by linker sequences within the *PR-1*$_{1294}$ promoter context. The genotypes of the analyzed plants are indicated. Values are means of the activities of the indicated number (#) of independent lines. LUC activities are expressed as relative light units (RLU) per μg of total protein. Error bars represent the SD of two independent experiments. wt, Wild type.

4B, lane 2). Apparently, the *LS4*-dependent pathway is not functional in this system. Probable explanations are that the WRKY factors driving this activation are not induced in time upon expression of NPR1-VP16 or, alternatively, that this activation mode requires additional sequences outside bp −813 to −573. Only residual activation was observed for *PR-1*$_{816–573}$*LS5*$_{mut}$ (Fig. 4B, lane 3) and to a lesser extent for *PR-1*$_{816–573}$ *LS7*$_{mut}$ (Fig. 4B, lane 4). Although some activation through *LS7* is detected, this experiment demonstrates that NPR1 can only efficiently activate the *PR-1*$_{816–573}$ promoter if both TGA-binding sites are present. Based on the previous data that NPR1 interacts with TGA factors (Zhang et al., 1999), we conclude that the activation observed in the transient assays is due to the direct association of NPR1 with TGA factors bound to *LS5* and *LS7* and that such a mechanism cannot operate on *LS4*.

Two TGA-Binding Sites Are Not Sufficient for NPR1-Mediated Activation

Two TGA-binding sites are not only found in NPR1-dependent, but also in NPR1-independent, SA-inducible promoters (Fode et al., 2008; Blanco et al., 2009). These promoters are inactive in the *tga256* mutant, indicating that the same clade of TGA factors is required for NPR1-dependent and NPR1-independent promoters. When fusing the promoter regions of two NPR1-independent (*GSTF8*, *IEGT38*) and two NPR1-dependent (*WRKY38*, *WRKY70*) genes upstream of the *PR-1* core promoter (−68 to +1), we observed transactivation by NPR1-VP16 only in the case of *WRKY38* and *WRKY70* (Fig. 5B). In contrast, all four promoters were activated by VP16-TGA2 in *tga256* mutant protoplasts (Fig. 5C). This analysis shows that only promoters that are under the control of NPR1 respond to

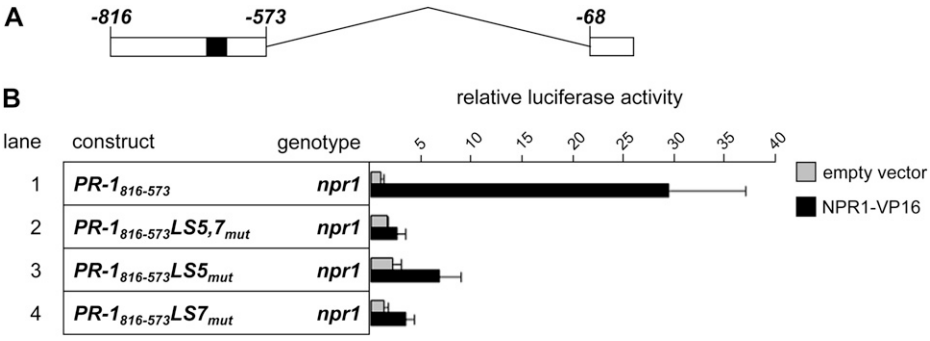

Figure 4. Importance of the cis-elements *LS5* and *LS7* for NPR1-VP16-mediated activation of the *PR-1*$_{816–573}$ promoter in *npr1* protoplasts. A, Schematic presentation of the *PR-1*$_{816–573}$ promoter. Sequences between bp −573 and −68 and upstream of bp −816 were removed. The black box depicts the relative position of the *LS5/LS7* motif. Mutations in *LS5* and *LS7* are as indicated in Figure 1. B, *PR-1*$_{816–573}$ activities in mesophyll protoplasts isolated from *npr1* plants. *PR-1*$_{816–573}$ promoter derivatives with mutations in *LS5*, *LS7*, or both elements were fused to the firefly *LUC* gene. Expression was analyzed in the presence of effector plasmids encoding NPR1 fused to the VP16 activation domain (black bars) under the control of the 35SC4PPDK promoter (Sheen et al., 1995). After transfection, protoplasts were incubated for 14 h in the presence of 5 μM SA. Relative LUC activities are expressed in arbitrary luminescence units, normalized to *Renilla* LUC activity. The *PR-1*$_{816–573}$ promoter activity in the absence of effectors was set to 1. Values are means of three replicates ± SD.

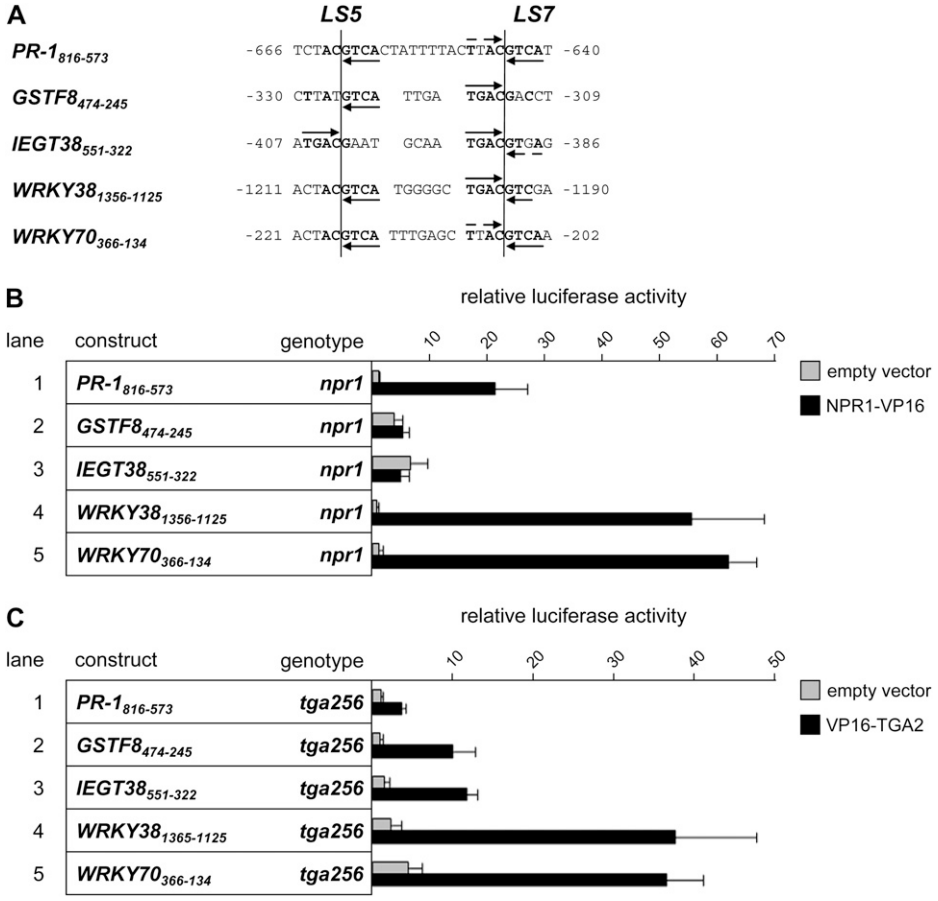

Figure 5. Trans-activation of SA-induced promoters by NPR1-VP16 in *npr1* protoplasts. A, Schematic presentation of the two TGA-binding sites within the four promoters (*GSTF8*, *IEGT38*, *WRKY38*, *WRKY70*) and their relative positions within their promoter contexts. Conserved positions with respect to the ideal TGA-binding site TGAC/GTCA are depicted in boldface letters; each TGAC half-site is marked by an arrow provided that 3 or 4 bp are in consensus with this motif. Vertical lines denote the centers of the palindromes. B, Activities of chimeric promoters in mesophyll protoplasts isolated from *npr1* plants. Upstream sequences of the promoters of *GSTF8*, *IEGT38*, *WRKY38*, and *WRKY70* were amplified and fused to the −68 *PR-1* promoter fragment so that the TGA-binding sites were in the same relative position to the TATA box as in $PR\text{-}1_{816–573}$. Promoter derivatives were fused to the firefly *LUC* gene. Expression was analyzed in the presence of effector plasmids encoding NPR1 fused to the VP16 activation domain (black bars) under the control of the 35SC4PPDK promoter (Sheen et al., 1995). After transfection, protoplasts were incubated for 14 h in the presence of 5 μM SA. C, The same promoters as in B were analyzed in *tga256* protoplasts in the presence and absence of TGA2 fused to the VP16 activation domain (black bars) under the control of the cauliflower mosaic virus *35S* promoter. Relative LUC activities are expressed in arbitrary luminescence units, normalized to *Renilla* LUC activity. The $PR\text{-}1_{816–573}$ promoter activities in the absence of effectors were set to 1. Values are means of three replicates ± SD.

NPR1-VP16 in our transient system. Based on our hypothesis that the activation through NPR1-VP16 is due to recruitment of the protein to TGA factors at the promoter region, we conclude that two TGA-binding sites are not sufficient for this type of activation.

Comparison of basal expression levels of the five promoters in the absence of the effector proteins revealed that their relative activities differed depending on the genotype of the protoplasts. The three NPR1-dependent promoters showed lower activities than the NPR1-independent promoters in the *npr1* mutant, whereas their activities were higher in the *tga256* mutant (Fig. 5, B and C). Thus, only those promoters that are targets of NPR1 can be subject to the repressive effect of TGA factors.

Comparison of the sequence covering the two TGA-binding sites in NPR1-dependent and NPR1-independent promoters has suggested that the number of base pairs between the palindromic centers might be a relevant feature: in NPR1-independent promoters, the spacing is conserved (12 bp; Krawczyk et al., 2002) and differs from the number of base pairs between the palindromic centers of the respective sites within the *PR-1* (17 bp), *WRKY38* (14 bp), and *WRKY70* (15 bp) promoters. Therefore, we tested whether changing the sequence between the palindromic centers of *LS5* and

LS7 to 12 bp would interfere with NPR1 recruitment. The sequence CTATTTTAC between *LS5* and *LS7*, which can be exchanged by linker sequences without affecting promoter activity (Lebel et al., 1998), was replaced by AAAA (Fig. 6A). In the transient assay system, the *PR-1*$_{816–573}$*LS5*$_{4xa}$*LS7* promoter was activated less efficiently by NPR1-VP16 than the corresponding promoter with the wild-type *LS5/LS7* motif (Fig. 6B). In order to investigate whether the spacing or the sequence between the two TGA-binding sites is the discriminating factor, we constructed *PR-1*$_{816–573}$*LS5*$_{9xa}$*LS7*, thus restoring the original spacing found in the *PR-1* promoter. However, this promoter was as inefficiently activated by NPR1-VP16 as *PR-1*$_{816–573}$*LS5*$_{4xa}$*LS7* (Fig. 6B, lane 3), indicating that the spacing between the two TGA-binding sites is not the only determining factor and that certain sequence requirements between the TGA-binding sites are relevant for NPR1 function.

Comparison of the expression data of *PR-1*$_{1294}$*LS5*$_{4xa}$*LS7* and *PR-1*$_{1294}$*LS5,7*$_{mut}$ in transgenic plants reveals that mutating the distance between the TGA recognition sites has a different effect than mutating both elements: *PR-1*$_{1294}$*LS5,7*$_{mut}$ shows a wild-type-like expression pattern (Fig. 2, lane 2), whereas *PR-1*$_{1294}$*LS5*$_{4xa}$*LS7* is severely compromised (Fig. 6C, lane 2). Obviously, the *LS4*-dependent pathway cannot be activated in *PR-1*$_{1294}$*LS5*$_{4xa}$*LS7*. Assuming that the activity of the *LS4*-dependent pathway is repressed by *LS5*, we can also explain why the activity of *PR-1*$_{1294}$*LS7*$_{mut}$ is com-

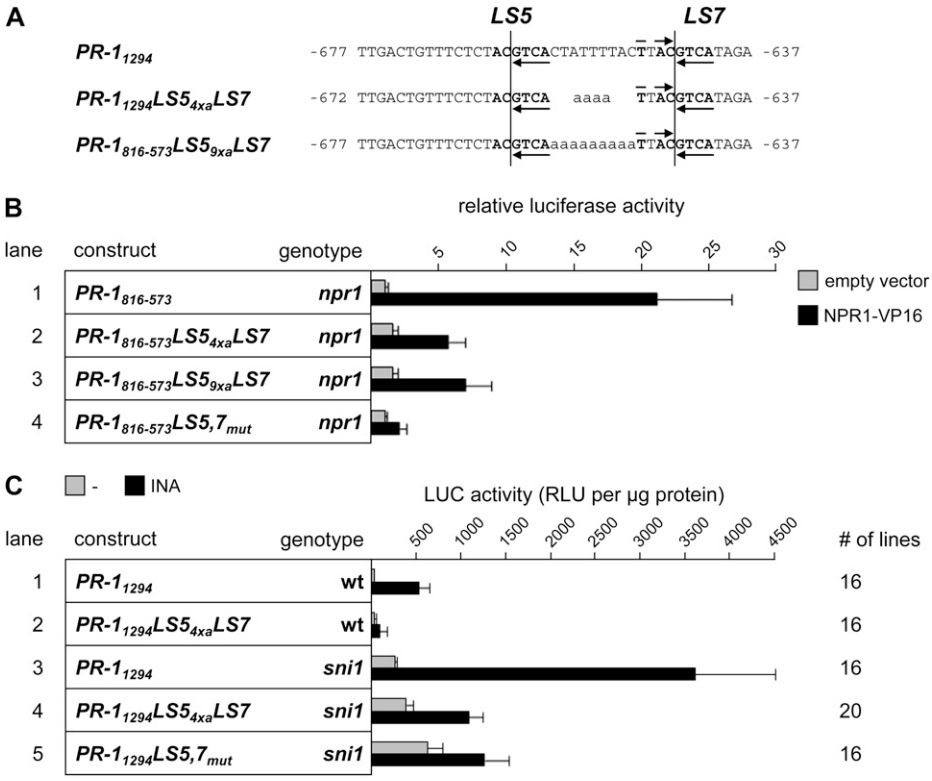

Figure 6. Influence of the number of base pairs between *LS5* and *LS7* on NPR1-VP16-mediated activation of the *PR-1*$_{816–573}$ and *PR-1*$_{1294}$ promoter activities. A, Mutations introduced into the *PR-1*$_{1294}$ promoter context to study the relevance of the number of base pairs between the two TGA-binding sites. The sequence CTATTTTAC between *LS5* and *LS7* was replaced by four and nine A residues, respectively. Conserved positions compared with the ideal palindrome TGAC/GTCA are depicted in boldface letters, and vertical lines denote the centers of the two palindromes. Each TGAC half-site is marked by an arrow provided that 3 or 4 bp are in consensus with this motif. B, *PR-1* promoter activities in mesophyll protoplasts isolated from *npr1* plants. Cells were transfected with the indicated plasmids. *PR-1* promoter derivatives that contain the sequences from −816 to −573 upstream of the *PR-1* core promoter (−68 to +1) fused to the firefly *LUC* gene were used as reporter constructs. Mutated cis-elements are indicated on the left. Expression was analyzed in the presence of effector plasmids encoding NPR1 fused to the VP16 activation domain (black bars) under the control of the 35SC4PPDK promoter (Sheen et al., 1995). After transfection, protoplasts were incubated for 14 h in the presence of 5 μM SA. Relative LUC activities are expressed in arbitrary luminescence units, normalized to *Renilla* LUC activity. The *PR-1*$_{816–573}$ promoter activity in the absence of effectors was set to 1. Values are means of three replicates ± SD. C, LUC activities of 2-week-old plants grown axenically on MS plates without (gray columns) and with (black columns) 30 μM INA were measured. The genotypes of the analyzed plants are indicated. Values are means of the activities of the indicated number (#) of independent lines. LUC activities are expressed as relative light units (RLU) per μg of total protein. Error bars represent the SD of two independent experiments. wt, Wild type.

promised: in this promoter, the *LS4*-dependent pathway is repressed by *LS5*, and the *LS5/LS7*-dependent pathway cannot operate because NPR1 functions through an intact *LS5/LS7* motif rather than through *LS7* alone.

In *sni1*, $PR\text{-}1_{1294}LS5_{4xa}LS7$ did not reach the induced expression levels of $PR\text{-}1_{1294}$ (Fig. 6C, lanes 3 and 4), thus resembling $PR\text{-}1_{1294}LS5,7_{mut}$ (Fig. 6C, lane 5). The reduced expression levels are most likely due to the fact that NPR1 cannot function through TGA factors at these promoters.

The *PR-1* Promoter Contains at Least Two Different Integration Sites for SNI1-Mediated Suppression

Next, we addressed the question of which promoter sequences are important for SNI1 to act as a repressor. Previously, it has been speculated that SNI1 negatively affects the *PR-1* promoter by targeting *LS4* (Kesarwani et al., 2007). This hypothesis was based on the findings that basal and induced levels of the *PR-1* promoter are elevated in *sni1* (Li et al., 1999), a phenomenon that has also been observed for a promoter lacking *LS4* (Lebel et al., 1998). Analysis of $PR\text{-}1_{1294}LS4_{mut}$ in wild-type and *sni1* plants indicated that SNI1 still influences *PR-1* in the absence of *LS4* (Fig. 7A).

In order to address the question of which sequences might be relevant for the repressive effect of SNI1, we analyzed $PR\text{-}1_{816–573}$, which contains 244 bp, including the sequences *LS4*, *LS5*, and *LS7* upstream of the −68 core promoter region (Fig. 4A), in *npr1*, *sni1*, and *sni1 npr1* mutant plants. The $PR\text{-}1_{816–573}$ promoter still mediated INA-inducible expression in the wild-type background, although the expression levels were lower than those of $PR\text{-}1_{1294}$ (Fig. 7B, lane 2). As observed for the $PR\text{-}1_{1294}$ promoter (Fig. 1C), expression of $PR\text{-}1_{816–573}$ required NPR1 (Fig. 7B, lane 3). As NPR1 is only necessary in the presence of SNI1 (Li et al., 1999), it is concluded that $PR\text{-}1_{816–573}$ contains either direct or indirect target sites for SNI1. This notion is supported by the observation that basal expression levels of $PR\text{-}1_{816–573}$ were enhanced in *sni1* (Fig. 7B, lane 4). However, whereas the $PR\text{-}1_{1294}$ promoter was induced to higher levels in *sni1* than in wild-type plants (Fig. 1C), the $PR\text{-}1_{816–573}$ promoter did not show this hyperinduction (Fig. 7B, compare lanes 2 and 4). Thus, sequences outside the −816 to −573 region are important for hyperinduced expression of the promoter in *sni1*. Whereas the $PR\text{-}1_{1294}$ promoter is still inducible in the *sni1 npr1* mutant background (Fig. 1C, lane 4), this is not the case for the $PR\text{-}1_{816–573}$ construct (Fig. 7B, lane 5). Apparently, this promoter fragment lacks sequence information that allows induction of the promoter in the absence of NPR1 and SNI1.

In summary, the analysis displayed in Figure 7 has several implications: first, regulatory sequences between −816 and −573 are sufficient for INA induction in wild-type plants and increased background expression in the absence of SNI1; second, regulatory sequences outside the −816 to −573 region are necessary for full promoter activity in wild-type plants, hyperinduction in *sni1*, and INA-induced expression in the *sni1 npr1* mutant background.

W Boxes Are Required for Full Promoter Activity in Wild-Type Plants and Hyperinduced Expression in the *sni1* Mutant Background

When searching for putative positive regulatory cis-elements outside the −816 to −573 region, we noticed two *W* boxes [(T)TGAC(T)] and one *W* box-like element [(C)TGAC(T)] spaced by approximately 20 bp between positions −550 and −480 (Fig. 8A). As transcription of many genes encoding WRKY factors is SA inducible (Dong et al., 2003; Wang et al., 2006), we

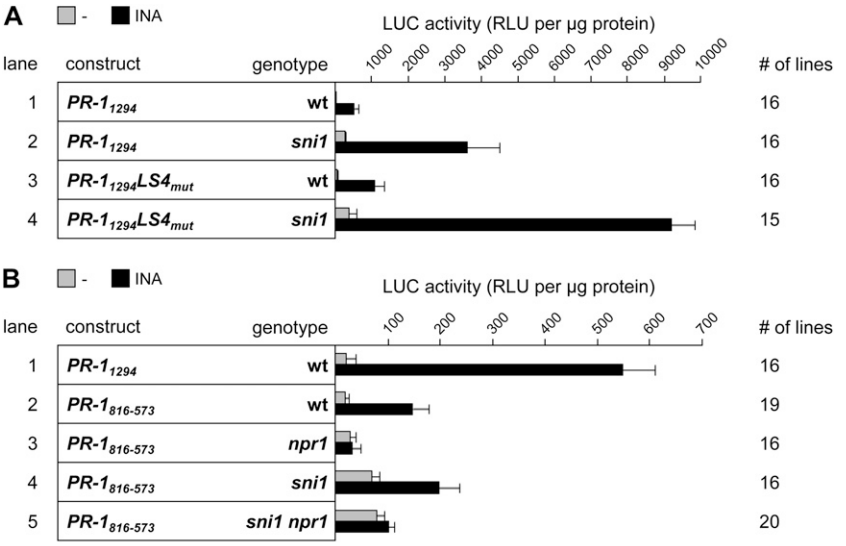

Figure 7. Identification of promoter regions containing SNI1 integration sites. A, Influence of *LS4* on the expression in *sni1*. B, Expression of $PR\text{-}1_{816–573}$ in *npr1*, *sni1*, and *sni1 npr1*. LUC activities of 2-week-old plants grown axenically on MS plates without (gray columns) and with (black columns) 30 μM INA were measured. The indicated *LS* elements were replaced by linker sequences within the $PR\text{-}1_{1294}$ or the $PR\text{-}1_{816–573}$ promoter context. The genotypes of the analyzed plants are indicated. Values represent means of the activities of the indicated number (#) of independent lines. LUC activities are expressed as relative light units (RLU) per μg of total protein. Error bars represent the SD of two independent experiments. wt, Wild type.

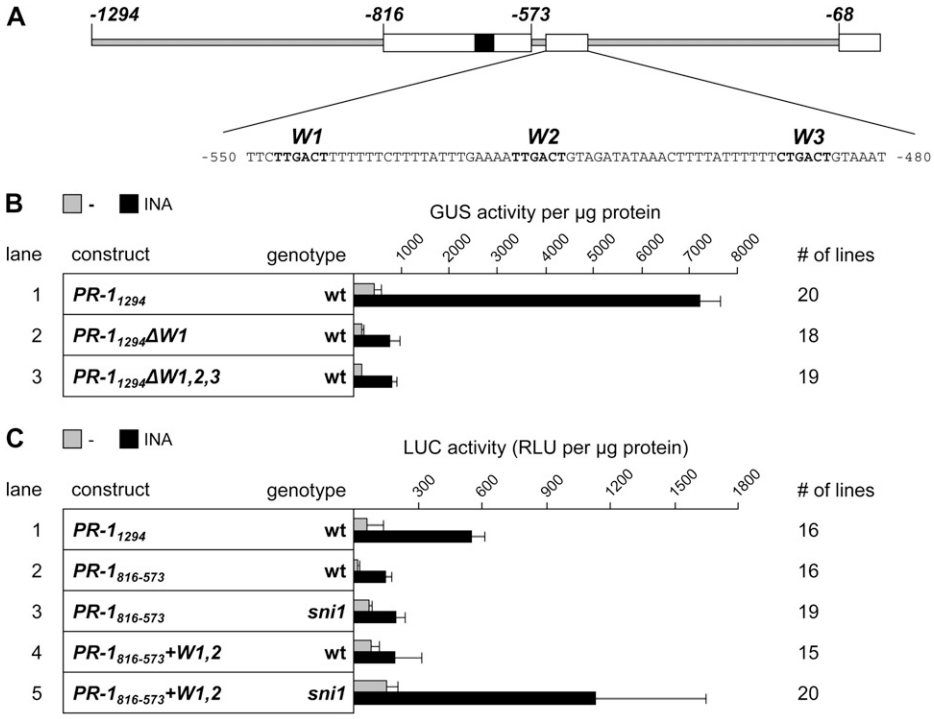

Figure 8. Influence of *W* boxes *W1*, *W2*, and *W3* on expression of the *PR-1* promoter. A, Schematic presentation of the "*W* box cluster" within the *PR-1* promoter. The small black box within the −816 to −573 region depicts the relative position of the *LS5/LS7* motif. The sequence between −550 and −480 containing the three *W* boxes is indicated. *PR-1*$_{1294}$*ΔW1* and *PR-1*$_{1294}$*ΔW1,2,3* contain deletions between bp −547 to −537 and −573 to −483, respectively. For constructing *PR-1*$_{816–573}$*+W1,2*, bp −572 to −509 were added to the *PR-1*$_{816–573}$ promoter. B, GUS activities of 2-week-old plants grown axenically on MS plates without (gray columns) and with (black columns) 30 μM INA were measured. Distinct mutations in the *W* box cluster were introduced into the *PR-1*$_{1294}$ promoter context. The genotypes of the analyzed plants are indicated. Values represent mean GUS activities (pmol methylumbelliferyl glucuronide mg^{-1} protein min^{-1}) of the indicated number (#) of independent lines. Error bars represent the SD of two independent experiments. C, LUC activities of 2-week-old plants grown axenically on MS plates without (gray columns) and with (black columns) 30 μM INA were measured. Sequences encoding two *W* boxes were added to *PR-1*$_{816–573}$. The genotypes of the analyzed plants are indicated. Values are means of the activities of the indicated number (#) of independent lines. LUC activities are expressed as relative light units (RLU) per μg of total protein. Error bars represent the SD of two independent experiments. wt, Wild type.

hypothesized that these boxes might contribute to *PR-1* promoter activity. Indeed, deletion of box *W1* within the *PR-1*$_{1294}$ promoter context reduced promoter activity (Fig. 8B, lane 2). Simultaneous deletion of *W1*, *W2*, and *W3* (Fig. 8B, lane 3) had the same effect as deletion of *W1* alone, indicating that *W2* and *W3* have no function in the absence of *W1*.

Next, we added sequences from –572 to –509, which contained the two canonical *W* boxes *W1* and *W2*, downstream of the *PR-1*$_{816–573}$ construct and investigated the activity of the resulting promoter *PR-1*$_{816–573}$ *+W1,2* in the wild-type and the *sni1* background. Whereas the added *W* boxes did not alter the general expression levels of *PR-1*$_{816–573}$ under inducing conditions in wild-type plants (Fig. 8C, lanes 2 and 4), the *PR-1*$_{816–573}$*+W1,2* construct showed hyperinduced expression in the *sni1* mutant background (Fig. 8C, lanes 4 and 5). Thus, hyperinduction in *sni1* does not only require NPR1 (Fig. 1C) and a functional *LS5/LS7* motif (Figs. 2 and 6C) but also *W* boxes downstream of −573.

DISCUSSION

Since expression of the *PR-1* gene correlates with the onset of the long-lasting plant immune response SAR, it has been used as a paradigm to analyze transcriptional control mechanisms that are operational in SAR leaves (Cao et al., 1994, 1997; Lebel et al., 1998; Zhang et al., 2003; Rochon et al., 2006; Kesarwani et al., 2007). The strong difference between basal and induced transcript levels is not merely due to the induced nuclear translocation of the coactivator NPR1 but is supported by the partially redundant negative functions of SNI1 and TGA factors in the noninduced state (Kesarwani et al., 2007). When trying to integrate data on the expression characteristics of the *PR-1* promoter in *npr1*, *sni1*, and *tga2 sni1* mutants with results obtained from previous promoter analysis (Lebel et al., 1998), the following model of the interplay of these regulatory proteins on the promoter had been proposed (Kesarwani et al., 2007; Supplemental Fig. S1).

The *W* box *LS4* functions as a target site of SNI1, *LS5* is the target site of the negative function of TGA2, and TGA factors at *LS7* function in concert with the coactivator NPR1. In this study, we specifically addressed the question of which sequences are important for the regulation of the *PR-1* promoter by negatively and positively acting TGA factors, SNI1 and NPR1. To this end, we analyzed *PR-1* promoter derivatives encoding different combinations of mutated *LS4*, *LS5*, and *LS7* elements in *sni1* and *npr1*. The updated working model is illustrated in Figure 9.

Direct NPR1-Mediated Activation Requires an Intact *LS5/LS7* Motif

Numerous studies have suggested that the key activator of *PR-1* expression, NPR1, functions through TGA factors at the cis-element *LS7* of the *PR-1* promoter (Lebel et al., 1998; Johnson et al., 2003; Rochon et al., 2006). Here, we provide evidence that NPR1 can activate the promoter either through a functional *LS5/LS7* unit or, in the absence of *LS5/LS7*, through *LS4*. Based on previous results that NPR1 activates *WRKY* promoters (Wang et al., 2006) and as it directly interacts with TGA factors (Zhang et al., 1999), we postulate that NPR1 activates the *LS4*-dependent pathway through activated WRKY factors, whereas the *LS5/LS7*-dependent pathway exploits the interaction between NPR1 and TGA factors at the promoter. In contrast to previous assumptions, *LS7* is not sufficient for the latter activation mechanism. This concept is supported by transient assays with a truncated promoter construct that showed that *LS4* alone, or *LS4* in combination with one TGA-binding site or two binding sites with an inappropriate intervening sequence, is not sufficient for efficient NPR1-VP16-mediated promoter activation (Figs. 4B and 6B). Simultaneous mutation of *LS4* and *LS5*, which have been previously characterized as negative elements, wipes out promoter activity in transgenic plants, indicating that *LS7* alone cannot support transcription (Fig. 3). Analysis of the *PR-1* promoter derivatives in *sni1* led to the same conclusions: $PR\text{-}1_{1294}$ activity was higher in *sni* than in *sni1 npr1* (Fig. 1C), indicating that NPR1 does not only interfere with the function of SNI1 but that it also functions as a transcriptional coactivator in the absence of SNI1. Full NPR1-dependent hyperactivation, which exceeds the expression levels of the induced promoter in wild-type plants, is not observed with promoter derivatives that either contain only one TGA-binding site (Fig. 2) or an inappropriate sequence between both sites (Fig. 6C).

Consistently, 21 out of 48 promoters that were candidates to be directly targeted by NPR1 (Wang et al., 2006) contain two TGA-binding sites (Supplemental Fig. S5). When cloning the respective sequences of two selected promoters (*WRKY38*, *WRKY70*) upstream of the *PR-1* core promoter, the resulting chimeric promoters were activated by NPR1-VP16 in transiently transformed protoplasts (Fig. 5B). Equivalent promoter constructs containing two TGA-binding sites from SA-inducible NPR1-independent promoters (*GSTF8*, *IEGT38*; Blanco et al., 2005) were not activated by NPR1-VP16. Comparison of the sequence around the TGA-binding sites in the NPR1-dependent promoters did not reveal any obvious pattern. Our transient assay system might allow the definition of the sequence requirements that are important for NPR1 function.

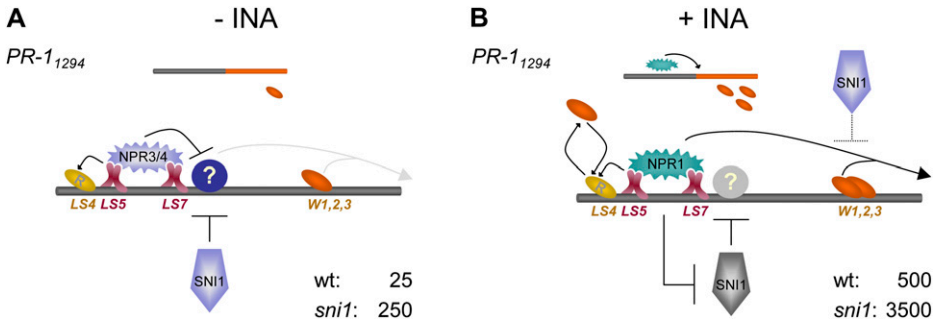

Figure 9. Mechanistic models showing the interplay of different regulatory factors at the *PR-1* promoter under noninduced (–INA) and induced (+INA) conditions. A, In the absence of INA, basal expression levels of the wild-type promoter are under the control of two partially redundant repression mechanisms. We assume that an activator at a yet unknown site between –816 and –573 is repressed by the *LS5/LS7* motif through the TGA2/NPR3/4 complex and by SNI1. A negative influence at *LS4* by negatively acting WRKY transcription factors (R) is favored by TGA factors at the *LS5* site. Under noninduced conditions, transcription of genes coding for activating WRKY transcription factors is low. Average LUC activities of the construct in the wild type (wt) and *sni1* are given in the right corner. B, In the presence of INA, NPR1 acts as a transcriptional coactivator by binding to the TGA factors at the *LS5/LS7* motif. The function of SNI1 and its target is inactivated. *LS4* remains occupied by negatively acting WRKY transcription factors. Positively acting WRKY factors can only be recruited in the absence of *LS5*, allowing the *LS5/LS7*-independent activation mode. Activating WRKY transcription factors, which are under the control of the SNI1/NPR1 regulon, contribute to high induction of the promoter by binding to WRKY boxes *W1* to *W3*. NPR1 synergistically interacts with WRKY transcription factors at *W1*, *W2*, and *W3*. Average LUC activities of the construct in the wild type and *sni1* are given in the right corner.

LS5 Interferes with *LS4*-Dependent Activation of the *PR-1* Promoter

The importance of *LS5* for the direct activation of the *PR-1* promoter by NPR1 was previously occluded by the observation that mutation of *LS5* did not compromise promoter activity (Lebel et al., 1998). The severely compromised activity of the $PR\text{-}1_{1294}LS4,5_{mut}$ promoter indicates that activation of $PR\text{-}1_{1294}LS5_{mut}$ is, like the activation of $PR\text{-}1_{1294}LS5,7_{mut}$, due to an activation pathway that depends on *LS4* (Figs. 2 and 3). In turn, the severely compromised activities of $PR\text{-}1_{1294}LS7_{mut}$ and $PR\text{-}1_{1294}LS5_{4xa}7$ indicate that the *LS4*-dependent pathway is repressed by *LS5* (Figs. 1B and 6C). A possible scenario is that TGA factors at *LS5* recruit repressive WRKY factors to *LS4*, which in turn would interfere with the access of positively acting WRKY factors (Fig. 9). Due to the complexity and redundancy of the WRKY transcription factor family, it seems almost impossible to define which WRKY transcription factors are responsible for the function of *LS4* (Xu et al., 2006; Eulgem and Somssich, 2007).

Other NPR1-dependent *PR* genes like *PR-2* and *PR-5* do not contain TGA-binding sites and thus are likely to be regulated indirectly through a mechanism that relies on the transcriptional activation of WRKY factors. Interestingly, these promoters can be activated by ectopic expression of WRKY70, whereas only an aberrantly long and faint *PR-1* mRNA is detected in these plants (Li et al., 2004). Likewise, ectopic expression of WRKY18 is not sufficient for the activation of *PR-1*, unless leaves get older (Chen and Chen, 2002). Thus, it might well be that *LS5* prevents the activation of *PR-1* by ectopically expressed WRKY factors and that such a mechanism is not operational in other *PR* promoters.

Repression of Basal *PR-1* Promoter Activity by TGA Factors Requires an Intact *LS5*/*LS7* Motif

It has been shown that TGA factors and SNI1 are partially redundant in repressing the *PR-1* promoter (Kesarwani et al., 2007). Thus, the importance of the cis-elements required for TGA-dependent repression can be best monitored in *sni1*. In *sni1*, mutation of *LS5* or *LS7* or both sites led to high basal expression levels (Fig. 2). As the two NPR1-related proteins NPR3 and NPR4 interact with TGA factors and as the *npr3 npr4* mutant shows increased basal levels of *PR-1* expression (Zhang et al., 2006), we speculate that repression through the *LS5/LS7* motif is mediated through these proteins (Fig. 9A). Like NPR1, NPR3 and NPR4 seem to require the intact *LS5/LS7* motif to be efficiently recruited. This notion is consistent with the observation that the two NPR1-dependent promoters of *WRKY* genes *WRKY38* and *WRKY70* have lower basal expression levels in the *npr1* mutant than the two NPR1-independent promoters *GSTF8* and *IEGT38*, whereas their expression levels are higher in the *tga256* mutant, which cannot recruit NPR proteins to promoter regions (Fig. 5, B and C). Thus, basal levels of *WRKY38* and *WRKY70* promoters might be repressed through NPR3 and NPR4 by the same mechanism as the *PR-1* promoter.

This model is consistent with the finding that the *tga2 sni1* double mutant shows higher *PR-1* expression levels in the absence of INA as compared with the respective single mutants (Kesarwani et al., 2007). We postulate that NPR3/4 is not recruited to the promoter if TGA2 is missing. However, a recent report showed that TGA2 forms oligomers in vitro and that these aggregates are resolved by NPR1 (Boyle et al., 2009). It was concluded that TGA2 oligomers repress the promoter. In our view, the association of NPR3/4 with TGA factors at an intact *LS5/LS7* motif has to be considered as an alternative explanation.

SNI1 Target Sequences for Repression of Basal Levels Are Located between bp −816 and −573

Based on the finding that the *W* box *LS4* acts as a negative element, it has been suggested that SNI1 exerts its negative effect either directly or indirectly at this site (Kesarwani et al., 2007). As $PR_{1294}LS4_{mut}$ is derepressed in the *sni1* mutant background (Fig. 7A), we suggest that SNI1 represses basal expression levels through an *LS4*-independent mechanism. The finding that basal levels of the $PR\text{-}1_{816–573}$ promoter are repressed by SNI1 (Fig. 7B) points at a functionally relevant SNI1 integration site within these 244 bp. As this promoter does not show any hyperinduction in *sni1* (Fig. 7B), we postulate that SNI1 influences a different mechanism in the induced state.

Negative Regulation of Induced *PR-1* Expression by SNI1 Requires *W* Boxes Downstream of Position −573

In contrast to the $PR\text{-}1_{1294}$ promoter, $PR\text{-}1_{816–573}$ did not show different induced expression levels in *sni1* (Figs. 1C and 7B). As extension of the $PR\text{-}1_{816–573}$ promoter by *W* boxes *W1* and *W2* reestablished hyperinduction in *sni1* (Fig. 8C), we conclude that SNI1 exerts a negative effect through these sites. This repression might be mediated either directly or indirectly by regulating the expression of cognate WRKY transcription factors. As hyperinduced expression in *sni1* depends on NPR1 (Fig. 1C) and an intact *LS5/LS7* motif (Figs. 2 and 6C), we postulate that hyperinduction in *sni1* is mediated by the synergistic action of these WRKY transcription factors with NPR1 (Fig. 9B).

Sequences Downstream of bp −573 Are Necessary for NPR1/SNI1-Independent Regulation of the *PR-1* Promoter

Apart from not showing a hyperinduction in *sni1*, $PR\text{-}1_{816–573}$ is also not induced in the *sni1 npr1* mutant (Fig. 7B). As *W* boxes *W1* and *W2* restore the hyperinduction in *sni1* (Fig. 8C), they might play a role in the induction process in *sni1 npr1*. This conclusion is supported by functional analysis of these *W* boxes in

the *PR-1*$_{1294}$*LS4,5*$_{mut}$ construct. This promoter is not a target of NPR1 because it lacks *LS4* and an intact *LS5/LS7* motif, which mimics the situation in *npr1*. However, it is inducible in *sni1* (Supplemental Fig. S6), thus resembling processes in the *sni1 npr1* mutant. Its induction is abolished upon removal of the three *W* boxes downstream of −573 (Supplemental Fig. S6), which is consistent with the hypothesis that these *W* boxes are likely important for induction in *sni1 npr1*. This finding has implications for unraveling the SNI1/NPR1-independent induction pathway that depends on RAD51D (Durrant et al., 2007).

CONCLUSION

The updated working model (Fig. 9) for the regulation of the *PR-1* promoter is based on an experimental approach that, to our knowledge, has not been taken to this extent before: multiple combinations of mutations in previously identified regulatory cis-elements have been tested in different mutant backgrounds. Previous results of single promoter mutants in the wild-type background have been misleading, because the regulation of the *PR-1* promoter is complicated by the fact that it is not only directly regulated by NPR1 and SNI1 but also indirectly by WRKY transcription factors that are subject to a similar regulatory node.

We conclude from our results that the promoter is repressed by SNI1 at yet unidentified target sites between positions −816 and −573 (Fig. 9A). This target site is redundantly regulated by TGA factors at the *LS5/LS7* motif, possibly through the action of the redundant proteins NPR3 and NPR4. *LS4* works as a negative element, potentially due to the recruitment of negative WRKY factors by TGA factors residing at *LS5*. *W* boxes downstream of bp −573 do not play a major role due to low expression of the cognate WRKY transcription factors in the noninduced state. Under inducing conditions, NPR1 functions as an inhibitor of SNI1, displaces NPR3/4, and acts as a transcriptional coactivator. As soon as the promoter is activated, the SNI1-regulated positive activity does not play a role any longer. NPR1 acts synergistically with WRKY transcription factors at *W* boxes downstream of bp −573. The activity of these factors is under the negative control of SNI1.

This study has yielded novel insights into the complex regulation of the *PR-1* promoter and provides a framework for the integration of future results. The finding that two TGA-binding sites are required for the recruitment of NPR1 to the promoter and for repression in the absence of SNI1 has implications for further efforts to advance our understanding of the mechanism of action of NPR1 as a transcriptional coactivator and of the mechanism of action of NPR3 and NPR4 as negative regulators of *PR-1* expression. Analysis of *PR-1*$_{816–573}$ promoter derivatives in wild-type and *sni1 tga2* plants might yield the cis-elements that are essential for high basal expression levels. This might be instrumental for unraveling the mechanism of action of SNI1. To elucidate the influence of the *sni1* allele under induced conditions, the expression of WRKY transcription factors in INA-induced *sni1* and *sni1 npr1* plants has to be studied. *WRKY* genes that are under the control of an NPR1-independent induction mode in *sni1* would explain the SNI1/NPR1-independent induction of the *PR-1* promoter and might serve as future targets to study the function of RAD51D (Durrant et al., 2007).

MATERIALS AND METHODS

Vector Construction

The *PR-1*$_{1294}$ promoter, which covers the sequence from −1,294 to +34 relative to the transcriptional start site, was amplified with primers P1 and P2 (for primer sequences, see Supplemental Table S1) from genomic DNA isolated from Arabidopsis (*Arabidopsis thaliana*) accession Columbia (Col-0) plants using the iProof high-fidelity PCR kit (Bio-Rad). The resulting PCR fragment was digested with *Sal*I and *Xba*I and cloned into pUC18-Entry2, which contains a multiple cloning site flanked by *Att*L recombination sites. Substitution and deletion constructs were obtained by overlap extension PCR. To this end, P1 and P2 were utilized separately in two iProof PCRs in combination with primers carrying the mutations of interest (P3–P22; Supplemental Table S1). The two different fragments yielded by the first PCRs were purified after separation on a 1% agarose gel and diluted 1:200. The two diluted fragments were used as templates in a second PCR, again employing the primers P1 and P2. The resulting PCR fragment contained the desired DNA modification and was digested with *Eco*91I (−816) and *Mph*1103I (−573; for modifications of *LS* motifs) or *Mph*1103I and *Bpi*I (−370; for deletions of W boxes) before ligation into pUC18-Entry2-*PR-1*, cut with the corresponding enzymes.

The *PR-1*$_{816–573}$ promoter construct was generated by first amplifying *PR-1* promoter sequences between positions −1,294 and +1 by using primers P1 and P21. The PCR fragment was cut with *Sal*I and *Xba*I and cloned into vector pUC18-Entry2 as described above. Subsequently, the region upstream of the *Eco*91I site at −816 was exchanged against bp +86 to +586 of the *Tn5*-encoded *Chloramphenicol Acetyl Transferase* (*CAT*) gene, with an additional arbitrary 24 bp inserted between the *CAT* gene and the 5′ end of the promoter. For PCR-mediated amplification of the *CAT* gene, the primer combination P23 and P25 was used on the template plasmid pTAX-CAT, which is a derivative of pGA582 (An, 1987). The deletion between −569 and −68 was created by ligation of the vector fragment pUC18-Entry2-CAT-PR-1 cut with *Eco*91I (−816) and *Hpa*I (−68) and the promoter fragments from *Eco*91I (−816) to *Nde*I$_{(fill\ in)}$ (−573). As the fill-in reaction of the *Nde*I overhanging ends had destroyed the *Mph*1103I restriction site, an overlap extension PCR was performed. Primers carrying the *Mph*1103I restriction sites were P26 and P27, and flanking primers were P1 and P23. The resulting plasmid pUC18-Entry2-PR-1$_{816–573}$ was cut with *Mph*1103I to insert an oligonucleotide (P28/P29) that extends the promoter from −573 to −510, thus adding the *W* boxes *W1* and *W2*. Plasmids containing modifications of the *LS* motifs within the *PR-1*$_{816–573}$ promoter context were generated by exchanging the wild-type *Eco*91I/*Mph*1103I fragment of *PR-1*$_{816–573}$ against the corresponding fragment generated in *PR-1*$_{1294}$. Cloning of the promoter regions of Arabidopsis genes *GSTF8*, *IEGT38*, *WRKY38*, and *WRKY70* was done by amplifying the corresponding sequences using primers P30/P31 (IEGT38), P32/P33, (GSTF8), P34/P35 (WRKY70), and P36/P37 (WRKY38) with attached *Eco*91I/*Mph*1103I sites and exchanging the *PR-1* sequences in pUC18-Entry2-PR-1$_{816–573}$. Introduced mutations were confirmed by sequencing.

Using the Gateway cloning system from Invitrogen, *PR-1* promoter derivatives were recombined into the binary destination vectors pBGWL7 and pBGWFS7 (Karimi et al., 2005) that contain the Gateway recombination sites upstream of the firefly *LUC* and *GUS* reporter genes, respectively. The backbone of the NPR1-VP16 effector construct for transient assays is described by Krawczyk et al. (2002): the coding region of TGA2.2 was replaced by the coding region of NPR1. In the empty vector control construct, the coding region was deleted. The VP16-TGA2 effector construct was generated using Gateway technology by cloning the coding region of TGA2 into the

pDONR201 vector (Invitrogen) and subsequent recombination into the destination vector pAlligator1 (Bensmihen et al., 2004). pAlligator1 served as an empty vector control.

Plant Material and Plant Transformation

Arabidopsis Col-0 was used. Mutant *tga256* (Zhang et al., 2003) was obtained from Y. Zhang (University of British Columbia, Vancouver), *sni1-1* and *sni1 npr1* were from X. Dong (Department of Biology, Duke University), and *npr1-1* was from the Norwich Arabidopsis Stock Centre. For measuring reporter gene activity of transgenic plants, approximately 200 F2 seeds of reporter gene lines were surface sterilized and sown on sterile MS plates containing 0.5 g L^{-1} MES. Plates contained 30 μM INA dissolved in dimethyl sulfoxide; control plants contained 15 μL L^{-1} dimethyl sulfoxide. Plants were grown for 18 d at 22°C under long-day conditions (14 h of light [70 μmol photons m^{-2} s^{-1}] and 10 h of dark) and 60% humidity. For generation of transgenic plants, binary plasmids were electroporated into *Agrobacterium tumefaciens* strain GV3101 (pMP90). The resulting agrobacteria were used to transform Col-0, *npr1-1*, *sni1-1*, *sni1 npr1*, and *tga256* mutant plants using the floral dipping method (Clough and Bent, 1998). For determining the recruitment of NPR1-VP16 or VP16-TGA2, 5 μg of reporter plasmids (pBGWL7 carrying different $PR\text{-}1_{816–573}$ derivatives) and 15 μg of the effector plasmids were transformed in Arabidopsis *npr1-1* and *tga256* protoplasts (Yoo et al., 2007). Protoplasts were incubated in washing and incubation solution (Yoo et al., 2007) supplemented with 5 μM SA for 16 h before harvest. To normalize for the experimental variability, 1 μg of the plasmid p70SRuc containing the *Renilla LUC* gene under the control of the cauliflower mosaic virus *35S* promoter was added to each sample.

LUC and GUS Assays

To determine LUC activities of transgenic lines, protein extracts from transgenic plants were prepared by application of 700 μL of 1× cell culture lysis reagent (Promega) to approximately 300 to 500 mg of ground frozen plant material. The samples were shaken in an Eppendorf mixer until the plant powder was completely dissolved. After centrifugation (10 min, 12,000 rpm, 4°C), 10 μL of protein extract was added to each well of a precooled Greiner Lumitrac 200 plate. The plate was subsequently incubated for 20 min in the dark at 4°C to avoid background illumination during the LUC activity measurement. The LUC measurements were started 5 to 10 min after insertion of the plate in the FLUOstar Ultima luminescence plate reader (BMG Labtech) by adding 50 μL of LUC substrate buffer (Promega) to each well. All measurements were performed under the same conditions, using a detection interval of 10 s and a relative gain value of 3,600. After detection of the emitted light, the values were normalized to the protein concentrations as determined with the BCA protein assay kit (Thermo Scientific). Quantitative GUS assays using 4-methyl-umbelliferyl-β-D-glucuronide (Sigma-Aldrich) as a substrate (Jefferson, 1989) were done on microtiter plates. The released fluorescence was measured with a Cyto Fluor Series 4000 plate reader (Perspective Biosystems). The total amount of protein was determined using the Bradford reagent (Carl Roth). Eighteen to 22 independent lines per construct were analyzed in one experiment. Supplemental Figure S7 documents exemplarily the variation between the lines, which is due to "position effects." For calculating the means, the highest and the lowest expressing lines were excluded. As described before (Lebel et al., 1998), averages of the mean values from two independent experiments are presented. LUC activities of transfected protoplasts were determined with the dual LUC reporter assay system from Promega using the TD20/20 luminometer from TurnerBiosystems.

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *PR-1* (At2g14610), *NPR1* (At1g64280), *GSTF8* (At2g47730), *IEGT38* (At4g34138), *WRKY38* (At5g22570), and *WRKY70* (At3g56400).

Supplemental Data

The following materials can be found in the online version of this article.

Supplemental Figure S1. Working model suggesting the regulatory functions of the cis-elements *LS4*, *LS5*, and *LS7* within the *PR-1* promoter (adapted from Kesarwani et al., 2007).

Supplemental Figure S2. Steady-state *PR-1* transcript levels in wild-type, *sni1*, and *sni1 npr1* plants.

Supplemental Figure S3. Activation of $PR\text{-}1_{816–573}$ by NPR1 and NPR1-VP16 in transiently transformed *npr1* protoplasts.

Supplemental Figure S4. Activation of $PR\text{-}1_{816–573}$ by NPR1-VP16 as a function of SA in transiently transformed *npr1* protoplasts.

Supplemental Figure S5. Identification of TGA-binding sites in promoters directly regulated by NPR1.

Supplemental Figure S6. Influence of *W* boxes on expression of the $PR\text{-}1_{1294}LS4,5_{mut}$ promoter in *sni1*.

Supplemental Figure S7. Expression of $PR\text{-}1_{1294}$ in independent transgenic lines.

Supplemental Table S1. List of primer sequences.

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