NPR1-Independent Activation of Immediate Early Salicylic Acid-Responsive Genes in *Arabidopsis*

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Salicylic acid (SA) is a key signal for the activation of defense genes in response to stress. The activation of late defense genes by SA, such as *PR-1*, involves the participation of the NPR1 protein. This protein acts as coactivator of the TGA factors that recognize *as-1*-like elements in the *PR-1* promoter. Considering that functional *as-1*-like elements are also found in the promoter of SA- and auxin-responsive immediate early genes, we tested the hypothesis that NPR1 is also required for activation of these genes. The expression of the immediate early genes glutathione *S*-transferase (*GST6*) and glucosyltransferase (*EIGT*) was studied in *npr1* mutant and wild-type *Arabidopsis* plants. In the *npr1* mutant background, SA and 2,4-dichlorophenoxyacetic acid were unable to promote transcription of *PR-1* but effectively stimulated the expression of *GST6* and *EIGT*. Furthermore, increased binding of proteins to the *GST6 as-1*-like promoter element was detected in nuclear extracts from *npr1* and wild-type plants after treatment with SA. In summary, these results indicate that activation of immediate early genes by SA proceeds through an NPR1-independent pathway. Therefore, we propose that activation by SA of immediate early and late genes occur by different mechanisms.

Salicylic acid (SA) plays an important role in the onset of defense reactions against environmental stressors in plants (Alvarez 2000; Borsani et al. 2001; Delaney et al. 1994; Rao and Davis 1999). The rise in SA levels triggers the activation of a number of defense genes (Maleck et al. 2000; Schenk et al. 2000). Among these genes, the best characterized code either for proteins with antimicrobial activity, such as pathogenesis-related (PR) proteins (Van Loon and Van Strien 1999) or for detoxifying enzymes, such as glutathione *S*-transferases (GST) and glucosyltransferases (GT). These enzymes play an important role in cell protection against oxidative stress (Edwards et al. 2000; Li et al. 2001). GST and GT are encoded by multigene families that are activated by pathogen infection (Alvarez et al. 1998; Horvath et al. 1998; Levine 1994; Pontier et al. 2001), different xenobiotics, and cellular stress-related agents, including SA, auxins, and H₂O₂ (Horvath and Chua 1996; Marrs 1996; Xiang et al. 1996).

Kinetic studies done in tobacco revealed the existence of two different groups of genes activated by SA, i.e., late and immediate early genes. Late genes such as *PR-1* are activated after several hours of treatment with SA, through a process dependent on de novo protein synthesis (Qin et al. 1994; Uknes

et al. 1993), and immediate early genes such as *GST1* (*GNT35*) (Xiang et al. 1996) and *IEGT* (immediate early-induced glucosyltransferase) (Horvath and Chua 1996) are activated after 30 min of treatment with SA or xenobiotics. Consistent with an immediate early response, the activation of *IEGT* by SA does not require de novo protein synthesis (Horvath and Chua 1996; Horvath et al. 1998).

Despite the difference in activation kinetics, a common SA-responsive element, named *activation sequence-1* (*as-1*), has been found to function in the promoter of both *PR-1a* (Strompen et al. 1998) and *GST1* tobacco genes (Droog et al. 1995). Involvement of *as-1*-like elements in the SA-induced activation of late and immediate early genes in species other than tobacco has also been described. This is the case of *PR-1* (Lebel et al. 1998) and *GST6* in *Arabidopsis* (Chen and Singh 1999; Chen et al. 1996). The involvement of *as-1*-like elements in positive and negative regulation of the *PR-1* gene by SA in *Arabidopsis* is further supported by the results of functional analysis of the *PR-1* gene by linker-scanning mutagenesis (Lebel et al. 1998).

as-1 is characterized by two imperfect TGACGTCA palindromic motifs that bind bZIP transcription factors of the plant TGA family (Krawczyk et al. 2002; Lam and Lam 1995; Xiang et al. 1997). The interaction between *as-1*-like elements and TGA factors is crucial to the regulation of *PR* and *GST* genes (Fan and Dong 2002; Johnson et al. 2001a; Niggeweg et al. 2000a; Pontier et al. 2001). This was mainly demonstrated using dominant negative mutants of TGA factors in tobacco (Niggeweg et al. 2000a; Pontier et al. 2001).

The interaction between *as-1*-like elements and TGA factors in the activation of late genes involves a novel protein named NPR1. In fact, *Arabidopsis* mutants in the NPR1 protein (*npr1*) are incapable of expressing the *PR* genes (*PR-1*, *PR-2*, and *PR-5*) and fail to develop pathogen resistance in response to SA or active analogs of SA (Cao et al. 1994). In addition, recent evidence supports the idea that the interaction of NPR1 with *Arabidopsis* TGA2 is promoted by SA and is essential for *PR-1* gene activation (Després et al. 2000; Fan and Dong 2002; Kinkema et al. 2000; Subramaniam et al. 2001).

Consistent with a role as a TGA factor coactivator, NPR1 has been shown to interact in vitro and in vivo with different members of the TGA family. This interaction has been documented for transcription factors from *Arabidopsis*, tobacco, tomato, and rice (Chern et al. 2001; Després et al. 2000; Fan and Dong 2002; Niggeweg et al. 2000b; Subramaniam et al. 2001; Zhang et al. 1999; Zhou et al. 2000). NPR1 has an ankyrin repeat domain suited for protein-protein interactions (Cao et al. 1997; Ryals et al. 1997) such as those reported above.

At present, it is not known if NPR1 participates in the activation of immediate early genes. This seems a reasonable

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possibility, since the activation of early genes, like that of late genes, involves the participation of both TGA factors (Johnson et al. 2001a; Niggeweg et al. 2000a; Pontier et al. 2001) and *as-1*-like elements (Chen and Singh 1999; Droog et al. 1995; Ulmasov et al. 1994).

Thus, in this paper, we evaluated whether, in *Arabidopsis*, the NPR1 protein is required for the activation of the immediate early genes glutathione *S*-transferase (*GST6*) and glucosyltransferase (*IEGT*) by SA and 2,4-dichlorophenoxyacetic acid (2,4-D), using *npr1* mutant plants. Results provide substantial evidence for the existence of different pathways in the activation of late and early genes by SA in *Arabidopsis*. While activation of *PR1*—a well-characterized late gene—proceeds through a NPR1-dependent pathway that is not activated by xenobiotics such as 2,4-D, activation of *GST6* and *IEGT*—two genes characterized here as early genes—proceeds through a NPR1-independent pathway that is also activated by 2,4-D.

RESULTS

To study the role of NPR1 in the activation of immediate early genes by SA, we selected *GST6* and *IEGT*. *GST6* (AT2g47730) is a member of the gene family coding for GST in *Arabidopsis*, in which a functional SA- and auxin-responsive *as-1*-like promoter element have been identified (Chen and Singh 1999). *IEGT* (AT4g34138; UGT73B3 according to Lim et al. 2002) was selected among *Arabidopsis* GT genes for its similarity with tobacco *IEGT* (Horvath and Chua 1996). An *as-1*-like element has been identified in the tobacco *IEGT* gene, which could explain its induction by SA and other xenobiotics (Horvath et al. 1998). An inspection of the putative promoter sequence of the *Arabidopsis* *IEGT* also shows the presence of an *as-1*-like element in position -529 to -509 from the ATG, although its role in expression remains to be elucidated. *PR-1* (AT2g14610), a late gene responsive to SA, was selected as a positive control for NPR1-dependent activation.

Fig. 1. Time course of expression of salicylic acid (SA)-activated genes in wild-type and *npr1* mutant *Arabidopsis* plants. **A**, Wild-type and **B**, *npr1* mutant *Arabidopsis* seedlings were treated with H₂O (C) or 0.5 mM SA (SA) for the indicated periods (in hours). RNA blots (20 µg total RNA per lane) were sequentially hybridized with ³²P-labeled probes for *GST6*, *IEGT*, *PR1*, and *actin* genes. **C**, through **E**, Comparison of the induction levels of *GST6*, *IEGT*, and *PR1* genes by SA in wild-type and *npr1* plants. Induction levels were calculated as follows. The mRNA level at each timepoint was quantified with a PhosphorImager and was calibrated with the corresponding loading control of actin mRNA. Then, the values from SA-treated samples were divided by the values of their corresponding control samples treated in the absence of SA. Data presented correspond to one representative experiment. This experiment was repeated at least three times and similar induction patterns were detected.

Time courses of activation of *GST6*, *IEGT*, and *PR-1* genes were analyzed in wild-type *Arabidopsis* seedlings treated with 0.5 mM SA for different periods. Control treatments were performed in the absence of SA. The mRNA levels of these genes were detected by Northern blot analysis (Fig. 1A) and were quantified with a phosphorImager (Fig. 1C, D, and E, white bars). Activation of *GST6* and *IEGT* genes by SA was detected after 1 h of treatment with SA and reached its peak value at 2 h (Fig. 1A, C, and D, white bars). In contrast, *PR-1* gene activation reached its peak value after 24 h of treatment (Fig. 1A and E, white bars). These results are consistent with the idea that both *GST6* and *IEGT* are early activated by SA, whereas *PR-1* belongs to the late-activated genes.

To assess whether the NPR1 protein is required for *GST6* and *IEGT* gene activation by SA, *Arabidopsis npr1* mutant seedlings were subjected to the same treatments. Results, shown in Figure 1B, indicate that, in *npr1* plants, *GST6* and *IEGT* genes were activated by SA to levels similar to those

seen in wild-type plants (Fig. 1A and B). In addition, the activation kinetics of these genes in the *npr1* mutant plants was similar to that in wild-type plants (Fig. 1C and D, compare white and gray bars). As expected, SA did not activate the *PR-1* gene in *npr1* seedlings (Fig. 1B and E). These results indicate that the NPR1 protein is not required for the activation of *GST6* and *IEGT* by SA.

To further investigate the mechanism of the immediate early response of *GST6* and *IEGT* to SA and to test whether it occurs without requiring de novo protein synthesis, we analyzed the expression of both genes in the presence of cycloheximide (CHX), a protein synthesis inhibitor. Wild-type and *npr1* *Arabidopsis* seedlings were pretreated for 1 h with or without CHX (20 μ g/ml), and then, were treated for 2 or 24 h with or without SA (0.5 mM). After these treatments, the steady state levels of *GST6*, *IEGT*, and *PR-1* mRNAs were quantified.

As previously shown in Figure 1, treatment of wild-type and *npr1* plants with SA for 2 h increased 2.4- to 4.8-fold the

Fig. 2. Effect of cycloheximide (CHX) on the expression of salicylic acid (SA)-activated genes in wild-type and *npr1* mutant *Arabidopsis* plants. **A**, Wild-type and **B**, *npr1* mutant *Arabidopsis* seedlings were pretreated for 1 h with H₂O (lanes 2 to 5) or 20 μ g of CHX per ml (lanes 6 to 9), and then, were treated for the indicated periods in the absence (lanes 2 to 3 and 6 to 7) or presence of 0.5 mM SA (lanes 4 to 5 and 8 to 9). Nontreated control is shown in lane 1. RNA blots (20 μ g of total RNA per lane) were sequentially hybridized with ³²P-labeled probes for *GST6*, *IEGT*, *PR1*, actin, and 18S rRNA genes. **C**, through **E**, Comparison of the induction levels of *GST6*, *IEGT*, and *PR1* genes in wild-type and *npr1* plants. Induction levels were calculated as follows. The mRNA level at each timepoint was quantified with a PhosphorImager and was calibrated with the corresponding loading control of 18S rRNA. Then, the values from SA-treated samples were divided by the values of their corresponding control samples treated in the absence of SA. Surprisingly, treatment with CHX decreased the actin mRNA levels in plants treated for 24 h with SA (lanes 7 and 9). Data presented correspond to one representative experiment. This experiment was repeated at least three times and similar induction patterns were detected.

GST6 and *IEGT* mRNAs levels (Fig. 2A and B [lanes 2 and 4], C, and D). In contrast, SA only increased the *PR-1* mRNA level (34-fold) after 24 h of treatment in wild-type but not in *npr1* plants (Fig. 2A and B [lanes 3 and 5] and E). Treatment with CHX had different effects in the mRNA steady state levels of *GST6*, *IEGT*, and *PR-1* genes. CHX alone for 2 h increased *GST6* mRNA to levels higher than those reached after 2 h of treatment with SA. The effect of CHX was not sustained nor further potentiated by cotreatment with SA (Fig. 2A and B [lanes 6 to 9] and C). In contrast, treatment with CHX alone for 24 h increased *IEGT* mRNA to levels similar to those seen after treatment with SA for 2 h (Fig. 2A and B [lane 4 compared with lane 7] and D). Treatment with both CHX and SA produced further increases in *IEGT* mRNA levels after 2 and 24 h of treatment (Fig. 2A and B [lanes 8 and 9] and D). Unlike *GST6*, *IEGT* mRNA levels remained elevated after CHX treatment. As reported (Qin et al. 1994), we detected no activation of *PR-1* gene in the presence of CHX, either alone or together with SA (Fig. 2A and B [lanes 6 through 9] and E).

The fact that CHX did not block the activation of *GST6* and *IEGT* indicates that this process does not require de novo protein synthesis. These results are consistent with the idea that—unlike *PR1*—*GST6* and *IEGT* form part of the immediate early response of plants to SA. Although *GST6* and *IEGT* are early-activated by SA and sensitive to CHX treatment, the observed differences in the response to CHX (Fig. 2C and D) suggest that *GST6* and *IEGT* mRNA levels are differentially regulated.

To determine if NPR1 plays a role in the activation of immediate early genes by compounds other than SA, we studied the activation of *GST6* and *IEGT* by the synthetic auxin 2,4-D. We chose this compound because it has been shown to activate *GST6* (Chen and Singh 1999; Chen et al. 1996). We evaluated the effect of 2,4-D on the expression of *GST6*, *IEGT*, and *PR-1* in wild-type and *npr1* *Arabidopsis* plants.

In wild-type plants, 2,4-D increased the mRNA levels of both *GST6* and *IEGT* (Fig. 3A, C, and D, white bars). This activation reached its peak value between 5 and 12 h of treatment. The mRNA levels remained elevated after 24 h of treat-

Fig. 3. Time course for expression of 2,4-dichlorophenoxyacetic acid (2,4-D)-activated genes in wild-type and *npr1* mutant *Arabidopsis* plants. **A**, Wild-type and **B**, *npr1* mutant *Arabidopsis* seedlings were treated with dimethyl sulfoxide (C) or 100 μ M 2,4-D (2,4 D) for the indicated periods (in hours). A control sample treated with 0.5 mM salicylic acid (SA) for 24 h was included. RNA blots (20 μ g of total RNA per lane) were sequentially hybridized with 32 P-labeled probes for *GST6*, *IEGT*, *PR1*, and actin genes. **C**, through **E**, Comparison of the induction levels of *GST6*, *IEGT* and *PR-1* genes in wild-type and *npr1* plants. Induction levels were calculated as follows. The mRNA level at each timepoint was quantified with a PhosphorImager and was calibrated with the corresponding loading control of actin mRNA. Then, the values from SA-treated samples were divided by the values of their corresponding control samples treated in the absence of SA. Data presented correspond to one representative experiment. This experiment was repeated at least three times and similar induction patterns were detected.

ment (Fig. 3A, C, and D, white bars). In *npr1* plants, 2,4-D was also able to activate *GST6* and *IEGT* genes (Fig. 3B, C, and D, gray bars). This activation was maximal at 5 h. In contrast, 2,4-D failed to activate *PR-I* in either wild-type or *npr1* plants (Fig. 3A, B, and E). In sum, these results indicate that NPR1 is not required for the activation of *GST6* and *IEGT* induced by 2,4-D.

As previously mentioned, a functional SA- and auxin-responsive *as-1*-like element has been identified in the *GST6* promoter (Chen and Singh 1999). We evaluated whether SA increases binding of nuclear factors to the *as-1* sequence in *npr1* and wild-type *Arabidopsis* plants. For this purpose, we analyzed the effect of SA on the binding of nuclear proteins to the *as-1* sequence of the *GST6* promoter, using nuclear extracts obtained from control and SA-treated *npr1* and wild-type plants. The ability of SA to increase the binding of proteins to the *as-1* sequence of the cauliflower mosaic virus (CaMV) 35S promoter was previously demonstrated, using total protein extracts from tobacco and *Arabidopsis* (Després et al. 2000; Jupin and Chua 1996) and nuclear protein extracts from tobacco (Stange et al. 1997).

For binding experiments, we prepared nuclear extracts from wild-type and *npr1* *Arabidopsis* seedlings treated with or without 0.5 mM SA for 2 h. The *as-1*-binding activity was detected by gel mobility shift assays, using as a probe a 41-bp DNA fragment of the *GST6* promoter that included the *as-1*-like element (*GST6-as-1* probe). SA increased the binding of nuclear proteins to the *GST6-as-1* probe in wild-type and in *npr1* plants (Fig. 4). To determine the specific binding, a nonradioactive *GST6-as-1* probe was used. Binding was competed for by an excess (18-, 90-, and 180-fold) of this probe but not by equivalent concentrations of a mutated *GST6-as-1* probe. The latter had three mutated bases in each of the imperfect TGACGTCA palindromic motifs. These results confirm the specificity of the binding (Fig. 4, lanes 4 to 9 and 13 to 18).

Fig. 4. Salicylic acid (SA)-activated binding of nuclear factors to the *GST6 as-1*-like promoter element in wild-type and *npr1* mutant *Arabidopsis* plants. Gel mobility shift assays were carried out with nuclear extracts obtained from wild-type and *npr1* mutant *Arabidopsis* seedlings treated for 2 h with H₂O (C) or with 0.5 mM SA (SA). A ³²P-labeled DNA fragment from the *GST6* promoter containing an *as-1*-like element (*GST6*) was used as probe. Lanes 1 and 10 show control reactions without nuclear extract. Competition experiments were performed with 18× (lanes 4 and 13), 90× (lanes 5 and 14), and 180× (lanes 6 and 15) molar excess of the wild-type probe or 18× (lanes 7 and 16), 90× (lanes 8 and 17), and 180× (lanes 9 and 18) molar excess of the mutated probe.

DISCUSSION

Results shown here provide substantial evidence for the existence of differences in the mechanism of activation of late and immediate early genes by SA in *Arabidopsis*. Unlike the activation of late genes, such as *PR1* that proceeds through a NPR1-dependent pathway, the activation of *GST6* and *IEGT*—two genes characterized here as early genes—does not require NPR1. In addition, late and early genes have different susceptibility to activation by xenobiotics, as shown by the fact that 2,4D activated the expression of *GST6* and *IEGT* but not that of *PR1*.

In the context of the disease defense response, several recent reports have indicated the existence of a SA-dependent and NPR1-independent pathway that leads to pathogen resistance (Clarke et al. 2000; Kachroo et al. 2000; Rairdan and Delaney 2002; Shah et al. 2001). For example, this pathway has been described in *Arabidopsis* mutants that have enhanced disease resistance, such as a constitutive expresser of *PR* genes (*cpr1*, *cpr5* and *cpr6*) (Clarke et al. 2000). Crossing of these mutants with the SA-deficient mutant *eds5* and the SA-degrading transgenic *nahG* plants resulted in a dramatic loss of resistance responses, indicating that SA is required to activate this pathway (Clarke et al. 2000). In contrast, crossing of *cpr* mutants with the *npr1* mutant did not affect resistance, indicating that NPR1 is not required (Clarke et al. 2000). Activation of this pathway requires, besides SA, an unknown signal produced by the pathogen and associated with components of the jasmonic acid and ethylene mediated pathways (Clarke et al. 2000; Dong 2001). Considering that SA per se is able to trigger systemic acquired resistance in wild-type plants, but is not able to restore pathogen resistance in the *npr1* mutant, it has been proposed that this second signal is associated with the local hypersensitive response (Dong 2001). In this context, it would be interesting to evaluate whether immediate early genes activated by SA by an NPR1-independent pathway, such as those described in this paper, could contribute to the SA-dependent and NPR1-independent pathway that leads to pathogen resistance.

Findings from this paper have important implications regarding the mechanism by which SA activates defense genes. Current genetic and molecular evidence supports the idea that the NPR1 protein plays an important role in the expression of the late *PR-I* gene activated by SA through its interaction with TGA factors that recognize *as-1*-like elements in the *PR-I* promoter (Dong 2001). Interestingly, TGA factors and *as-1*-like promoter elements have also been implicated in the SA- and auxin-activated expression of immediate early genes such as tobacco *GST1* (Chen and Singh 1999; Droog et al. 1995; Johnson et al. 2001a). Our finding that the NPR1 protein is not required for SA-induced activation of *GST6* and *IEGT* genes adds further support to the idea that the mechanism underlying the activation of immediate early genes is different from the one involved in the activation of late genes, such as *PR-I*. Thus, despite the fact that SA activates early and late genes using a common family of transcription factors and promoter-responsive elements, the activation of these groups of genes proceeds by different mechanisms.

Three different subclasses of TGA factors (I, II, and III), have been described according to their sequence similarities (Niggeweg et al. 2000b; Xiang et al. 1997). Conceivably, the difference in the mechanism of activation of early and late genes could derive from differences in the subclass of TGA factors that recognize the *as-1*-like elements in early and late genes. This hypothesis is supported by experimental evidence showing that immediate early and late genes are activated by TGA factors belonging to different subclasses. For example, there is evidence for the participation of TGA1a (a member of

subclass I) in the SA- and xenobiotic-induced activation of early genes in tobacco (Johnson et al. 2001b; Pascuzzi et al. 1998). Furthermore, in vivo 2,4-D-inducible binding of TGA1a to the *as-1*-like element of the tobacco *GST1* promoter has recently been demonstrated using chromatin immunoprecipitation assays (Johnson et al. 2001a). On the other hand, evidence suggests that TGA2 (a member of subclass II) is involved in the activation of late genes by SA in *Arabidopsis*. The use of an in vivo chimera reporter system in *Arabidopsis* plants showed that DNA binding and transcription mediated by the TGA2 factor is activated by SA but not by 2,4-D or other xenobiotics (Fan and Dong 2002). Interestingly, these effects were not observed in *npr1* mutant plants (Fan and Dong 2002).

The idea that different members of the TGA family can be activated by different mechanisms is supported by experiments showing differences in the ability of members of the TGA family to interact with the NPR1 protein. Members of subclass I, such as TGA1a from tobacco (Niggeweg et al. 2000b) and TGA1 or TGA4 from *Arabidopsis* (Després et al. 2000; Zhou et al. 2000), are unable to bind the NPR1 protein in in vitro protein-protein interaction assays. In contrast, substantial evidence indicates that members of subclass II from tobacco, *Arabidopsis*, and rice interact strongly with NPR1 in vitro (Chern et al. 2001; Després et al. 2000; Niggeweg et al. 2000b; Zhang et al. 1999; Zhou et al. 2000). Furthermore, in vivo interaction of *Arabidopsis* TGA2 with NPR1 has been recently reported (Fan and Dong 2002; Subramaniam et al. 2001). The idea that *as-1*-like elements from immediate early and late genes are targets for different TGA factors is also supported by the occurrence of differences in the relative distances between the two TGACGTCA palindromic cores in the *as-1*-like element from *GST* and *PR* genes (Krawczyk et al. 2002).

It has been shown that the NPR1 protein is required for SA-activated *PR* genes expression (Cao et al. 1994). Current evidence indicates that the NPR1 protein is involved in the mechanism by which SA activates TGA2 factor (Després et al. 2000; Fan and Dong 2002; Kinkema et al. 2000; Subramaniam et al. 2001). In contrast, our results clearly indicate that NPR1 is not required for the SA- and 2,4-D-activated expression of the immediate early *GST6* and *IEGT* genes in *Arabidopsis*. Consistently, we found that NPR1 is not required for the SA-activated binding of endogenous factors to the *as-1*-like element of the *GST6* promoter. We did not demonstrate in this work that TGA factors are part of the protein complex that bound to the *GST6-as-1* probe. Nevertheless, above-mentioned evidence from the literature of participation of TGA factors in the SA- and xenobiotic-induced activation of early genes in tobacco (Johnson et al. 2001a and b; Pascuzzi et al. 1998) support this idea. Furthermore, evidence that TGA factors are main components of the *as-1*-binding activity detected in protein extracts from tobacco and *Arabidopsis*, such as that used in this work, has also been provided by different groups (Jupin and Chua 1996; Lam and Lam 1995; Niggeweg et al. 2000a and b; Pontier et al. 2002). This evidence and our results are consistent with studies showing that the TGA factors involved in the NPR1-independent pathway do not interact with NPR1. All these studies support the idea that different members of the TGA family can be activated by different mechanisms.

Concerning the regulation of TGA factors of subclass I, the existence of a nuclear 120-kDa protein that interacts with the tobacco TGA1a in a stimulus-reversible way has been reported. It has been suggested that this protein could act as a repressor of gene expression (Johnson et al. 2001b). How SA or other stimuli can overcome this repression in order to trigger the expression of early genes is still not known. Evidence from our laboratory and other groups points to the involvement of redox signals and protein phosphorylation events in the activa-

tion of TGA factors (Garretón et al. 2002; Hidalgo et al. 2001; Jupin and Chua 1996; Stange et al. 1997). Further studies aimed at identifying the possible targets of phosphorylation and redox modification will allow a better understanding of the mechanisms involved in this pathway.

Although *GST6* and *IEGT* genes were found to behave as immediate early genes in response to SA, they differed in their response to CHX (Fig. 2C and D). Differences in behavior of early response genes to SA and CHX have been previously described in tobacco by Horvath and associates (1998). These authors classified genes into four groups, based on their mechanisms for the regulation of steady state levels of mRNAs. According to this classification, *Arabidopsis IEGT* belongs to class I, but *GST6* does not fit into any of the four classes described. With regard to the difference in response to CHX, different mechanisms of regulation have been invoked (Horvath et al. 1998). A sustained increase in mRNA levels in response to CHX, such as seen for *IEGT*, can be explained by the requirement of a labile protein for a negative regulation, either at the level of transcription or mRNA stability. A transient increase in mRNA levels, such as that detected for *GST6*, can be due to a direct effect of CHX on the signaling pathway. Interestingly, a recent report demonstrates that the levels of different TGA factors and their relative contribution into the protein complexes that bind *as-1* sequence can be regulated by proteolysis during development in *Arabidopsis* (Pontier et al. 2002). It would be interesting to determine if a mechanism of this kind may contribute to regulation of the transcriptional activity by TGA factors during defense and, thus, explain the effect of CHX.

Using nuclear extracts of wild-type and *npr1 Arabidopsis* plants, we found that SA potentiated the binding of endogenous factors to the SA- and auxin-responsive *as-1*-like element of the *GST6* promoter. The same experimental system has been previously used to study the effect of SA in tobacco (Jupin and Chua 1996; Stange et al. 1997) and *Arabidopsis* (Després et al. 2000). However, using total extracts from *npr1* plants, Després and associates (2000) were unable to demonstrate SA-induced changes in the binding of endogenous TGA factors to the CaMV 35S *as-1* element. Based on this finding, the authors concluded that the DNA-binding enhancement of TGA factors induced by SA depends on the presence of NPR1 protein (Després et al. 2000). Although we do not know the reason for this discrepancy, it may relate to the differences in the probes (CaMV 35S *as-1* vs. *GST6 as-1*) and preparations used (total vs. nuclear extracts).

Another aspect that must be considered in the future to understand the involvement of a common family of promoter-responsive elements in the activation of early and late genes, is the interaction of *as-1*-like elements with other promoter elements. In this context, it has been proposed that the W box, which binds factors from the WRKY family, could act as a SA-responsive element in the activation of late *PR* genes (Maleck et al. 2000). This is based on the fact that W boxes are present in the promoters of 31 genes that are coexpressed with the *PR-1* gene in SA-treated *Arabidopsis* plants, as shown by analysis of data from cDNA microarray experiments (Maleck et al. 2000). We are currently evaluating the presence of other putative SA-responsive elements in immediate early genes coexpressed with *GST6* in SA-treated *Arabidopsis*, to assess possible interactions of *as-1* with other elements in these promoters.

MATERIALS AND METHODS

Plant material and growth conditions.

Arabidopsis thaliana ecotype Columbia was used as the wild type in this study. The *npr1-1* mutant was obtained from X.

Dong (Duke University) (Cao et al. 1994). Seeds were sterilized in a 50% hypochlorite solution for 7 min, were washed three times with water, and were germinated on Murashige-Skoog (MS) medium containing 15 g of sucrose per liter. Seedlings were grown in vitro for 15 days in a growth chamber (22 $\pm$ 2°C, 16 h light).

Treatment of plants with SA, 2,4-D, and CHX.

Arabidopsis seedlings (15 days old), maintained in darkness during the last 12 h previous to the assay, were used in all experiments. To assay gene expression by Northern blot, 50 seedlings were used for each treatment. To assay the *as-1*-binding activity, 100 to 150 seedlings were used for each treatment. Seedlings were carefully taken out from the MS medium and were placed, root side down, in a petri dish containing 25 ml of a 0.5-mM SA or a 100- μ M 2,4-D solution. Stock solutions of SA (Sigma, St Louis or Riedel-deHaën, Seelze, Germany) and 2,4-D (Sigma) were fresh-prepared in H₂O and dimethyl sulfoxide (DMSO), respectively. Final concentration of DMSO in the medium was 0.5% (vol/vol). Control samples were incubated in water for SA treatments or in 0.5% vol/vol DMSO for 2,4-D treatments. Treatments were carried out in a growth chamber under constant temperature (22 to 25°C) and light. To test the effect of CHX, seedlings were first incubated for 1 h in a solution of 20 μ g of CHX per ml or in water as a control, and then, were treated for 2 or 24 h in the absence or in the presence of 0.5 mM SA. Stock solution of 10 mg of CHX per ml (Sigma) was fresh-prepared in water. Immediately after treatments, seedlings were frozen in liquid nitrogen and were stored at -70°C.

RNA extraction and Northern analysis.

Total RNA was extracted from frozen plant tissue samples using the TRIzol reagent (Invitrogen, Carlsbad, CA, U.S.A.), according to the manufacturer's protocol. Samples containing 20 μ g of RNA were separated on formaldehyde-agarose gels. After transfer of RNA onto nylon membranes (Hybond N; Amersham Biosciences, Corp. Piscataway, NJ, U.S.A.), filters were hybridized with the ³²P-labeled probe (about 50 $\times$ 10⁶ counts per minute [cpm]). For *GST6*, hybridization was carried out at 55°C, as described by Zhang and associates (1993). For *PR1*, *IEGT*, and 18S rRNA, hybridization was performed at 42°C in a buffer containing 6 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5 $\times$ Denhardt's solution, 50% formamide, 0.5% sodium dodecyl sulfate (SDS), 1 mM EDTA, and 150 μ g of salmon sperm carrier DNA per ml. For the actin gene, hybridization was performed as for *PR1*, *IEGT*, and 18S rRNA, except 40% formamide was used. Then, filters were washed at 42°C in 2 $\times$ SSC, 0.1% SDS for 10 min and twice at 55 to 60°C in 0.2 $\times$ SSC, 0.1% SDS for 10 min (*GST6*, *PR1*), 7 min (*IEGT*), 30 min (actin), or 90 min (18S rRNA).

Polymerase chain reaction (PCR)-amplified DNA fragments of 557 and 420 bp for *Arabidopsis GST6* and *PR1* genes, respectively, were obtained using previously described synthetic primers (Chen et al. 1996; Ho and Yang 1999). As a probe for *Arabidopsis IEGT*, a 360-bp DNA fragment was obtained by PCR, using as a template the expressed sequence tag (EST) ATTS 3278 and the 5' and 3' primers 5' GTGATCATCAT-CATCGAAAGC 3' and 5' GGA GTTTCTCAAGCTGGTC 3', respectively. For the *Arabidopsis* actin gene, a 700-bp DNA fragment, obtained by PCR using the 5' and 3' primers 5'-GCTATGTATGTCGCCATTCAAGC 3' and 5'-CATCATAT-TCTGCCTTTGC(A/G)ATCC 3', respectively, was used as a probe. For the 18S rRNA gene, a 1,100-bp DNA fragment was obtained by PCR using the EST H8B10T7 cloned in pBluescript SK⁻ as a template and the primers T7 and RP. The amplified fragments were sequenced and labeled by PCR, except

18S rRNA probe, which was labeled by random priming using [α -³²P]dCTP (RTS-RadPrime; Life Technologies, Gaithersburg, MD, U.S.A.).

Nuclear extracts and gel mobility shift assays.

Nuclear extracts were prepared from *Arabidopsis* seedlings according to the procedure described by Green and associates (1989), modified as follows. Frozen seedlings (4 g fresh weight) were ground in a mortar with liquid nitrogen and were resuspended in 30 ml of homogenization buffer (1 M hexylene glycol, 10 mM PIPES/KOH, pH 7.0, 10 mM MgCl₂, 10 mM NaF, 10 mM KCl, 1 mM EDTA, 0.5% vol/vol Triton X-100, 1 mM 2-mercaptoethanol, 0.8 mM phenylmethylsulfonyl fluoride [PMSF]). The homogenate was filtered through two layers of nylon and one of Miracloth, and chloroplasts were lysed by addition of 1/20 volume of NIB-T buffer (10 mM Tris-HCl, pH 9.5, 10 mM EDTA, 100 mM KCl, 0.5 M sucrose, 10% vol/vol Triton X-100, 4 mM spermidine, 1 mM spermine, 1 mM 2-mercaptoethanol) (Bouchez and Camilleri 1998). Then, the nuclei were sedimented at 2,000 $\times$ g for 10 min at 4°C, using polypropylene tubes to improve pellet recovering. The pellet was then washed twice with 10 ml of nuclei wash buffer (0.5 M hexylene glycol, 10 mM PIPES/KOH, pH 7.0, 10 mM MgCl₂, 10 mM NaF, 10 mM KCl, 1 mM EDTA, 1 mM 2-mercaptoethanol, 0.8 mM PMSF). Then, the nuclei were sedimented again at 2,000 $\times$ g for 10 min at 4°C. The nuclear pellet was resuspended in 400 μ l of lysis buffer (15 mM HEPES/KOH, pH 7.5, 110 mM KCl, 5 mM MgCl₂, 10 mM NaF, 0.5 mM EDTA, 2 μ g of leupeptin per ml, 1 mM benzamidine). After 1 h at 4°C, ammonium sulfate was added, and fractionation was performed as described (Green et al. 1989). After the second fractionation, the pellet was resuspended in 40 μ l of nuclear extract buffer (25 mM HEPES/KOH, pH 7.5, 50 mM NaCl, 0.1 mM EDTA, 10% [vol/vol] glycerol, 10 mM NaF, 2 μ g of leupeptin per ml, 1 mM benzamidine), was incubated for 10 min at 4°C, and was dialyzed against the same buffer overnight at 4°C, and was stored at -70°C. Protein concentration was determined using the BioRad kit (Bio-Rad, Hercules, CA, U.S.A.). The final yield was 60 to 90 μ g of nuclear protein per gram of fresh leaf tissue weight.

DNA protein binding assays were carried out with nuclear protein extracts (5 to 20 μ g of protein), incubated with the ³²P-labeled probe (25,000 cpm, 0.031 pmol) in 20 μ l of binding buffer (50 mM HEPES, pH 7.9, 130 mM NaCl, 2 mM MgCl₂, 9.5 mM dithiothreitol, 4% [vol/vol] glycerol and 15 ng of poly(dG)-poly(dC) [Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.]) for 5 min at room temperature. For competition experiments, the indicated molar excess of the nonradioactive probe was included in the binding assay 5 min before addition of the labeled probe. DNA protein complexes were separated from the unbound probe by electrophoresis in a 5% polyacrylamide gel in Tris-borate-EDTA buffer. Following electrophoresis, gels were dried and subjected to autoradiography at -70°C for 10 h. To obtain the *GST6 as-1* probe, the following oligonucleotide was used: 5'-GCTTCTCAGAGGAGGTCGTCATCAATGACATAAGTTCTAG-3' (protein binding motifs are indicated in bold). The complementary strand was synthesized using the primer 5'-GAGAAGC-3', dNTPs, (α -³²P)dCTP, and Klenow DNA polymerase, following standard protocols (Ausubel et al. 1997). For competition, nonradioactive *GST6 as-1* probe was obtained using the oligonucleotide 5'-GCTTCTCAGAGGAGGTCGTCATCAATGACATAAGTTCTAG-3' annealed to 5'-CTAGAATCTTATGTCATTGATGACGACCTCCTCTGAGAAGC-3', and a mutated *GST6 as-1* probe was prepared using the oligonucleotide 5'-GCTTCTCAGAGGAGGTCAGCGTCAACGCTATAAGAT

TCTAG-3' annealed to 5'-CTAGAATCTTATAGCGTTGAC
GCTGACCTCTCTGAGAAGC-3' (mutated nucleotides are
underlined).

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