

A Nuclear Casein Kinase 2 Activity Is Involved in Early Events of Transcriptional Activation Induced by Salicylic Acid in Tobacco¹

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Salicylic acid (SA) activates immediate early transcription of genes controlled by a family of DNA promoter elements named *as-1*-like elements. These elements are functional in the promoter of glutathione *S*-transferase genes. We have previously shown that SA increases the binding of tobacco (*Nicotiana tabacum* cv Xanthi nc) nuclear factors to the *as-1* sequence in a process mediated by protein phosphorylation. In this study we give evidence for the participation of a nuclear protein kinase CK2 (casein kinase 2) in the pathway activated by SA in tobacco. The first line of evidence comes from the evaluation of the CK2 activity in nuclear extracts prepared from tobacco plants treated with SA or water as a control. Results from these experiments indicate that SA increases the nuclear CK2 activity. The second line of evidence derives from the evaluation of the *in vivo* effect of 5,6-dichloro-1-(β -D-ribofuranosyl) benzimidazole (DRB), a cell-permeable CK2 inhibitor, on the responsiveness of the *as-1* sequence to SA. Results from these experiments indicate that DRB impairs the activating effect of SA on the transcription of both, the *GUS* reporter gene controlled by a tetramer of the *as-1* element, and the endogenous *gnt35* gene encoding a glutathione *S*-transferase, in transgenic tobacco plants. DRB also impaired the increasing effect of SA on the binding of nuclear factors to the *as-1* element. Furthermore, transcription of the *as-1/GUS* reporter gene activated by the synthetic auxin 2,4-dichlorophenoxyacetic acid and by methyl jasmonate was also inhibited by DRB. To our knowledge, this is the first report in which activation of a CK2 enzyme by a plant hormone is reported.

The transcription of defense genes induced by pathogens in plants is regulated by a complex network of signaling pathways (Yang et al., 1997; Scheel, 1998). In these pathways, metabolites like salicylic acid (SA), jasmonates, H₂O₂, ethylene, and nitric oxide, have been identified as secondary signals (Durner et al., 1997; Dong, 1998; Reymond and Farmer, 1998). At different times after pathogen recognition, these secondary signal substances accumulate transiently and transduce the signal into the nucleus where the transcription of specific defense genes is activated. The SA-mediated pathway is probably one of the most studied pathways in defense reactions (Durner et al., 1997; Dong, 1998; Reymond and Farmer, 1998). Important components of the SA-mediated signaling pathway have been identified by the use of molecular, biochemical, and genetical approaches (Durner et al., 1997; Zhang and Klessig, 1997; Zhang et al., 1999). Nevertheless, the molecular mechanism by which SA activates gene transcription is still poorly understood.

According to their activation kinetics, the genes directly activated by SA can be classified into two groups (Durner et al., 1997; Reymond and Farmer,

1998). One group corresponds to acidic pathogenesis-related (*PR*) genes, whose activation by SA requires *de novo* protein synthesis and forms part of the late events of the SA-mediated pathway (Uknes et al., 1993; Qin et al., 1994). A second group corresponds to genes whose activation by SA does not require protein synthesis and forms part of the immediate early events of the SA-mediated pathway. The genes coding for glutathione *S*-transferases (GSTs) are one of the best characterized (Liu and Lam, 1994; Xiang et al., 1996). They belong to the second group of SA-activated genes according to their activation kinetics and are involved in the detoxification of cytotoxic compounds produced during the defense reaction (Marrs, 1996). Furthermore, other genes, whose role in defense is not clear, have also been characterized as immediate early genes activated by SA (Horvath and Chua, 1996; Yang and Klessig, 1996; Horvath et al., 1998). It is interesting that some of the immediate early genes activated by SA can also be activated by other pathogenesis-related signals, such as methyl jasmonate (MeJA), H₂O₂, and also by auxins (Horvath and Chua, 1996; Xiang et al., 1996). Therefore, it seems that these early events represent a point of convergence of different signal transduction pathways.

Our interest has been focused in the elucidation of the mechanism by which SA activates transcription of immediate early genes. For this purpose we use a defined promoter *cis*-element which is responsive to

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SA, auxins, and MeJA (Qin et al., 1994; Zhang and Singh, 1994; Xiang et al., 1996). This *cis*-element belongs to the family of *as-1*-related sequences (Lam et al., 1989), characterized by two TGACG motifs for the binding of bZIP transcription factors of the TGA/OCS-binding factor (OBF) family (Foster et al., 1994). This element was first characterized in the 35S promoter from the cauliflower mosaic virus (CaMV) and in the promoter of opine synthase genes from the T-DNA of *Agrobacterium tumefaciens* (Bouchez et al., 1989; Lam et al., 1989). It is interesting that *as-1*-like sequences have been found in the promoter of *GST* genes where they control the transcriptional activation of these genes by SA, auxins, and MeJA (Ulmasov et al., 1994; Droog et al., 1995; Xiang et al., 1996; Chen and Singh, 1999).

Previous studies have given some information concerning the mechanism by which SA and other signals activate *as-1*-like elements. In vivo assays using transgenic plants indicate that gene transcription controlled by *as-1*-like elements does not require de novo protein synthesis (Liu and Lam, 1994; Qin et al., 1994), suggesting that post-translational modifications of premade proteins are involved. In vitro DNA-protein binding assays using either total (Jupin and Chua, 1996) or nuclear protein extracts (Stange et al., 1997) indicate that SA produces an increase in the binding of tobacco (*Nicotiana tabacum* cv Xanthi nc) transcription factors to the *as-1* sequence. This effect of SA was reversed by treatment of the extracts with alkaline phosphatase, suggesting the involvement of a protein phosphorylation event (Jupin and Chua, 1996; Stange et al., 1997). In support of this idea, we have previously shown that in vitro phosphorylation of nuclear proteins by an endogenous protein kinase (PK) activity increases the *as-1* binding activity, mimicking the effect of SA on entire plants (Stange et al., 1997). This effect of phosphorylation was inhibited by quercetin, but not by H-7 (1-[5-isoquinoliny]sulfonyl]-2-methylpiperazine) or genistein (Stange et al., 1997; Ramírez and Holuigue, unpublished data). It is known that these compounds inhibit different PK activities. Whereas quercetin inhibits PK CK2 and Tyr PK (Meggio et al., 1986), H-7 inhibits CAMP-dependent PK (PKA), CGMP-dependent PK (PKG), and PKC (Hidaka et al., 1984) and genistein inhibits Tyr PK (Akiyama et al., 1987). Therefore, the observation that only quercetin was able to inhibit the activating effect of protein phosphorylation on DNA binding supports the idea that a nuclear CK2 kinase may participate in the SA-induced phosphorylation of proteins that probably underlies the enhanced binding activity of *as-1* to nuclear factors. Furthermore, recent reports indicate that auxin potentiates the trans-activation capacity of a tobacco TGA factor (Pascuzzi et al., 1998). Therefore, transcription of genes controlled by the *as-1* element could be activated by increasing either the DNA binding activity or the trans-activation capacity of the nuclear factor.

In this study we give experimental evidence for the participation of a nuclear CK2 in the SA-mediated pathway leading to the activation of gene transcription controlled by the *as-1*-like element in tobacco. Treatment of tobacco plants with SA increases the nuclear CK2 activity. 5,6-Dichloro-1-(β -D-ribofuranosyl) benzimidazole (DRB), a cell-permeable CK2 inhibitor, consistently prevents the activating effect of SA on both the binding of nuclear factors to the *as-1* sequence, and the transcription of genes controlled by the *as-1* sequence (*as-1/GUS* and *gnt35* genes). Furthermore, our results show that DRB also inhibits the effect of 2,4-dichlorophenoxyacetic acid (2,4-D) and MeJA on the transcription of the *as-1/GUS* gene. Taken together, these observations support a more general role for CK2 in the *as-1* mediated activation of immediate early genes. Possible target proteins and mechanisms of CK2 regulation by SA are discussed.

RESULTS

SA Increases Nuclear CK2 Activity in Tobacco Plants

To study the participation of a nuclear kinase CK2 in the SA-mediated pathway, we first evaluated the effect of SA on the endogenous nuclear CK2 activity. For this purpose, we assayed the CK2 activity in nuclear extracts prepared from tobacco leaves treated with 1 mM SA or water for 1 h. The peptide RRRD-DDSDDD, which contains the consensus phosphorylation sequence for CK2 (Kuenzel and Krebs, 1985; Pinna, 1990), was used as the phosphate acceptor substrate. Either [γ -³²P]ATP or [γ -³²P]GTP was used as phosphate donors since CK2 is the only PK able to use either nucleotide as phosphate donor (Pinna, 1990). Results shown in Figure 1A indicate that a significant increase in CK2 activity, measured with GTP (2-fold, $P < 0.005$) and ATP (3.3-fold, $P < 0.05$), was detected after SA treatment. This effect of SA on the CK2 activity was detected using a relative broad range of protein extract concentrations (2.5–10 μ g of protein). Within this range CK2 activity increased linearly with protein concentration (data not shown).

To give further support to the idea that the PK activity detected corresponds to a CK2, the effect of the specific inhibitors heparin and DRB was assayed. Heparin is known for its specificity and efficiency as inhibitor of the CK2 enzyme (Hathaway et al., 1980). DRB and other halogenated benzimidazole derivatives specifically inhibit CK2 enzyme, less efficiently CK1 enzyme, but not other types of PKs (Meggio et al., 1990; Szyszka et al., 1995). Results shown in Figure 1B indicate that heparin (20 μ g mL⁻¹) strongly inhibited the kinase activity detected in extracts from water- and SA-treated plants (by 88% and 94%, respectively). Due to the specificity of this inhibitor (Hathaway et al., 1980), this heparin-sensitive kinase activity can confidently be attributed to a CK2. Unlike heparin, DRB (60 μ M) afforded only a partial inhibition of the kinase activity detected in extracts

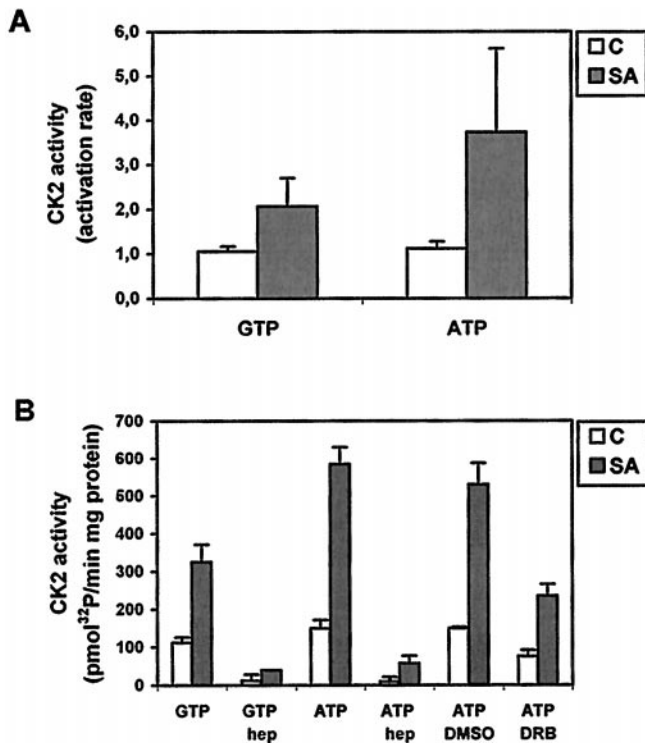


Figure 1. SA increases nuclear CK2 activity in tobacco leaves. A, CK2 activity was assayed in nuclear extracts obtained from leaves of tobacco plants treated with water (C, white bars) or with 1 mM SA (SA, gray bars) for 1 h. The peptide RRRDDDSDDD (1 mM) was used as phosphate acceptor and [γ - 32 P]GTP or [γ - 32 P]ATP (200 μ M) was used as phosphate donor, as indicated in the figure. Values are expressed as the activation rate between control and SA-treated samples and represent mean \pm SD of three independent extract preparations. Differences in CK2 activity between SA-treated and control samples were significant (GTP, *t* test, $P < 0.005$; ATP, *t* test, $P < 0.05$). B, Effect of DRB (60 μ M) and heparin (hep, 20 μ g mL $^{-1}$) on the CK2 activity in nuclear extracts obtained from leaves of tobacco plants treated with water (C, white bars) or with 1 mM SA (SA, gray bars) for 1 h. Control treatments for DRB effect included 0.5% (v/v) DMSO. CK2 specific activity is expressed as picomoles of 32 P incorporated in the substrate peptide min $^{-1}$ mg $^{-1}$ protein. Values are mean \pm SD of three determinations from the same extract.

from water- and SA-treated plants (50% and 56%, respectively; Fig. 1B). The inability of DRB to fully inhibit the CK2 activity in tobacco nuclear extracts is not surprising because only a partial inhibition of purified CK2 enzyme from different species has been reported using DRB (60–100 μ M; Meggio et al., 1990; Szyszka et al., 1995).

We also evaluated the effect of SA on the kinetic constants of the nuclear CK2 activity. For this purpose, the CK2 activity present in nuclear extracts from water- and SA-treated plants was measured using a broader range of peptide substrate concentrations (0.05–2 mM). As shown in Table I, SA increased the V_{\max} value (from 205 ± 14 to 571 ± 54 pmol 32 P incorporated min $^{-1}$ mg $^{-1}$ protein) without affecting the apparent K_m value (51 ± 0.02 and 48 ± 0.02 μ M for SA and water treatments, respectively).

This result suggests that SA increased the enzymatic activity without affecting the substrate affinity.

SA-Induced Binding of Nuclear Factors to the *as-1* Element Is Inhibited by DRB

To assess the participation of a CK2 in the SA-activated pathway for transcription of genes controlled by the *as-1* element, we evaluated the in vivo effect of CK2 inhibition on the SA-induced increase of the binding of nuclear factors to the *as-1* element. DRB was used to inhibit CK2 in in vivo experiments because this, unlike heparin, is a cell-permeable compound (Kim and Kahn, 1997; Delalande et al., 1999). In these in vivo experiments we used a higher concentration of DRB (500 μ M) as has been reported to be effective in other in vivo systems (Kim and Kahn, 1997; Delalande et al., 1999). Leaves from tobacco plants were pre-incubated for 30 min in the presence or absence of 500 μ M DRB; then SA was added to a final concentration of 1 mM and the incubation was continued for another 30 min. A control sample was incubated in water for 1 h. Addition of 0.5% (v/v) dimethyl sulfoxide (DMSO) did not have any effect on the control *as-1* binding activity (data not shown). Nuclear extracts were prepared from these samples as described in "Materials and Methods." The *as-1* binding activity in these extracts was detected by gel mobility shift assay using a 36-bp DNA fragment containing one copy of the *as-1* sequence as a probe. In agreement with our previous results (Stange et al., 1997), treatment of tobacco leaves with SA produced a 3.1-fold increase in the *as-1* binding activity (Fig. 2, lanes 2 and 3). Similar levels of increase in the *as-1* binding activity have been detected after 60 or 90 min of treatment with SA (Stange et al., 1997; data not shown). In the presence of DRB, SA elicited a considerably smaller increase in the *as-1* binding activity (1.9-fold the control activity; Fig. 2, lanes 3 and 4). This result is consistent with the participation of a CK2 in the activation by SA on the *as-1* binding activity.

Table I. Kinetic constants for CK2 activity from nuclear extracts of water- and SA-treated plants

Initial rates of CK2 with the peptide RRRDDDSDDD (0.05–2 mM) as the variable substrate were determined as described in "Materials and Methods." To calculate the kinetic constants, data were fit to the Michaelis-Menten equation by a nonlinear regression using SigmaPlot for Windows Version 4.0 (SPSS Inc). SE is given. Differences in K_m values were not significant (Student's *t* test, $P < 0.95$).

Nuclear Extract	V_{\max}	app K_m
	pmol min $^{-1}$ mg $^{-1}$ prot	μ M
C	205 ± 14	51 ± 0.02
SA	571 ± 54	48 ± 0.02

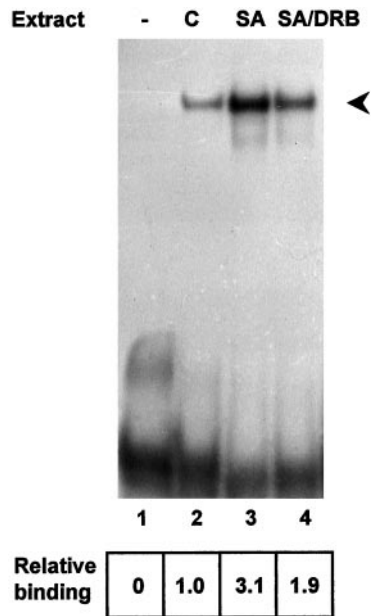


Figure 2. Binding of nuclear factors to the *as-1* sequence induced by SA is inhibited by DRB. Gel mobility shift assays were carried out with nuclear extracts from leaves of tobacco plants subjected to the following treatments: water for 1 h (C, lane 2); pre-incubation for 30 min with 0.5% (v/v) DMSO, then addition of 1 mM SA (SA, lane 3); and pre-incubation for 30 min with 500 μ M DRB, then addition of 1 mM SA (SA/DRB, lane 4). A 32 P-labeled DNA fragment containing one copy of the *as-1* sequence was used as probe. Lane 1 shows a control reaction without nuclear extract. The arrowhead indicates DNA-protein complexes. The relative binding activities, estimated by densitometric analysis of the autoradiography, are indicated below the figure.

SA-Activated Transcription of Genes Controlled by *as-1* Element Is Inhibited by DRB

To further assess the possible participation of a CK2 activity in the SA-activated pathway, we evaluated the effect of DRB on the SA-mediated activation of the gene transcription controlled by the *as-1* sequence. For this purpose, the activity of the β -glucuronidase (GUS) reporter protein was assayed in transgenic tobacco plants containing the $(as-1)_4/GUS$ chimeric gene after SA treatment in the presence or absence of DRB. Leaf discs from transgenic tobacco plants were pre-incubated for 30 min with 500 μ M DRB or 0.5% (v/v) DMSO. Then SA (1 mM) was added and incubation was continued for 5 h. Control samples were incubated in water during 5.5 h in the presence of 500 μ M DRB or 0.5% (v/v) DMSO. Total protein extracts were prepared from these samples, and GUS enzymatic activity was measured by a fluorometric assay. Treatment of tobacco leaves with SA significantly increases GUS activity (3.3-fold; $P < 0.001$; Fig. 3A). When treatment with SA was carried out in the presence of DRB, GUS activity was significantly reduced to 43% of the activity detected in the absence of DRB ($P < 0.001$; Fig. 3A). Similar results were obtained using an independent $(as-1)_4/GUS$ transgenic tobacco clone (data not shown).

We also evaluated the effect of DRB on the SA-mediated activation of the transcription of the endogenous *gnt35* gene. *gnt35* is one of the tobacco *GST* genes for which SA-, auxin-, and MeJA-induced transcription controlled by an *as-1* element has been reported (Droog et al., 1995; Xiang et al., 1996). Tobacco leaf samples were pretreated for 30 min with 500 μ M DRB (or 0.5% [v/v] DMSO in the control sample) and then for 5 h in the presence or absence of 1 mM SA. Total RNA was isolated from these samples and the level of *gnt35* and actin mRNA (constitutive control) were detected by northern-blot analysis. Treatment with SA increased the level of *gnt35* mRNA (4.6-fold the basal level normalized by actin signal; Fig. 3B,

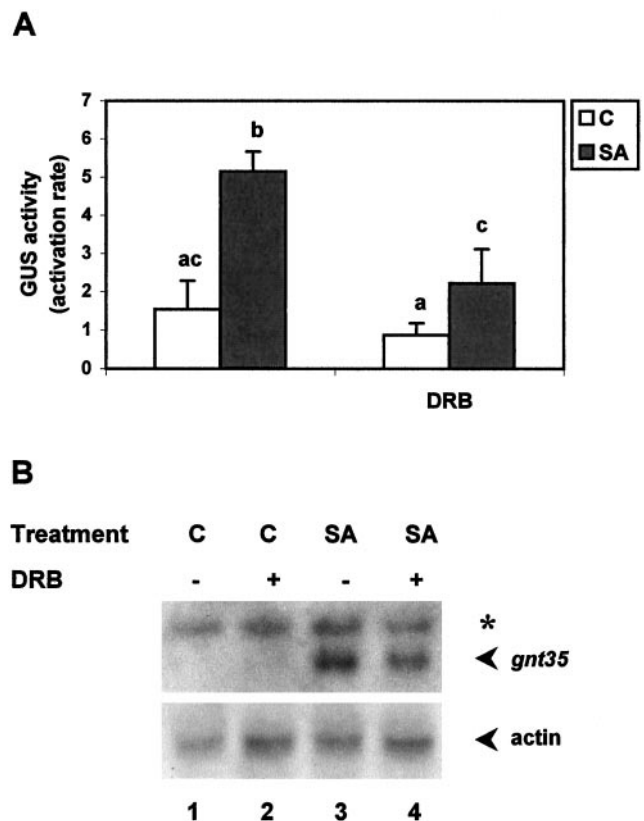


Figure 3. Expression of $(as-1)_4/GUS$ and *gnt35* genes induced by SA is inhibited by DRB. Leaf discs obtained from tobacco plants transformed with the $(as-1)_4/GUS$ gene were treated for 30 min with 500 μ M DRB (or 0.5% [v/v] DMSO in control samples) and then for 5 h in the presence or absence of 1 mM SA. A, GUS activity measured in protein extracts obtained from samples treated with SA (SA, gray bars) or water (C, white bars). Values are expressed as the activation rate between control and SA-treated samples and represent mean \pm SD of four independent experiments (each done in triplicate). Different letters indicate significantly different values ($P < 0.05$; ANOVA followed by Tukey HSD's test). B, Levels of *gnt35* mRNA and actin mRNA (constitutive control) detected by northern hybridization, using total RNA (20 μ g per lane) isolated from leaves subjected to the indicated treatments. Specific 32 P-labeled *gnt35* and actin gene fragments were used as probes. Transcript levels were estimated by densitometric analysis of the autoradiography. Asterisk, Signal from unknown nature detected with the *gnt35* probe.

lanes 1 and 2). DRB markedly inhibited the effect of SA on the level of *gnt35* mRNA (2.6-fold the basal level normalized by actin signal; Fig. 3B, lanes 3 and 4). Therefore, the inhibitory effect of DRB was specific for transcription of the SA-activated (*as-1*)₄/*GUS* and *gnt35* genes and did not affect transcription of the constitutive actin gene. This effect of DRB can be attributed to the inhibition of an endogenous CK2 activity.

Transcription of the (*as-1*)₄/*GUS* Gene Activated by Auxin and MeJA Is Inhibited by DRB

Because the *as-1* element is also responsive to auxins and MeJA, we evaluated whether DRB also inhibited the transcription of the (*as-1*)₄/*GUS* gene activated by the synthetic auxin 2,4-D and by MeJA. For this purpose, leaf discs from transgenic tobacco plants were treated with 2,4-D (100 μ M) or MeJA (50 μ M), in the presence or absence of DRB (500 μ M). After 5 h of treatment, GUS activity was measured in leaf samples. Treatment of tobacco leaves with 2,4-D (Fig. 4A) produced a significant increase in GUS activity (4.8-fold, $P < 0.001$). Treatment of tobacco leaves with MeJA (Fig. 4B) also produced a significant increase in GUS activity (2.1-fold, $P < 0.005$). In the presence of DRB, the GUS activity was significantly reduced to 50% ($P < 0.01$) and 49% ($P < 0.005$) of the activity detected with 2,4-D or MeJA alone. Similar results were obtained using an independent (*as-1*)₄/*GUS* transgenic tobacco clone (data not shown). These results suggest that a CK2 activity may also be involved in the MeJA- and auxin-mediated transcriptional activation of genes controlled by *as-1*-like elements in tobacco.

The partial inhibition exerted by DRB in the *in vivo* assays for transcriptional (Figs. 3 and 4) and DNA binding activities (Fig. 2) can be explained by our *in vitro* experiments (Fig. 1B) showing that DRB also exerts a partial inhibitory effect on CK2 activity.

DISCUSSION

Mechanism of Gene Activation by SA: Possible Role of CK2

In this study we give evidence for the involvement of a PK CK2 in the mechanism by which SA activates the transcription of certain immediate early genes. Thus, we showed that DRB, a cell-permeable CK2 inhibitor, impairs the activating effect of SA on the binding of nuclear factors to the *as-1* element and on the transcription of genes controlled by this cis-element (the (*as-1*)₄/*GUS* reporter gene and the endogenous *gnt35* gene). We also showed that DRB impairs transcription of the (*as-1*)₄/*GUS* reporter gene activated by 2,4-D and MeJA. These results from *in vivo* experiments support previous evidence obtained from *in vitro* experiments where the involvement of protein phosphorylation events in the acti-

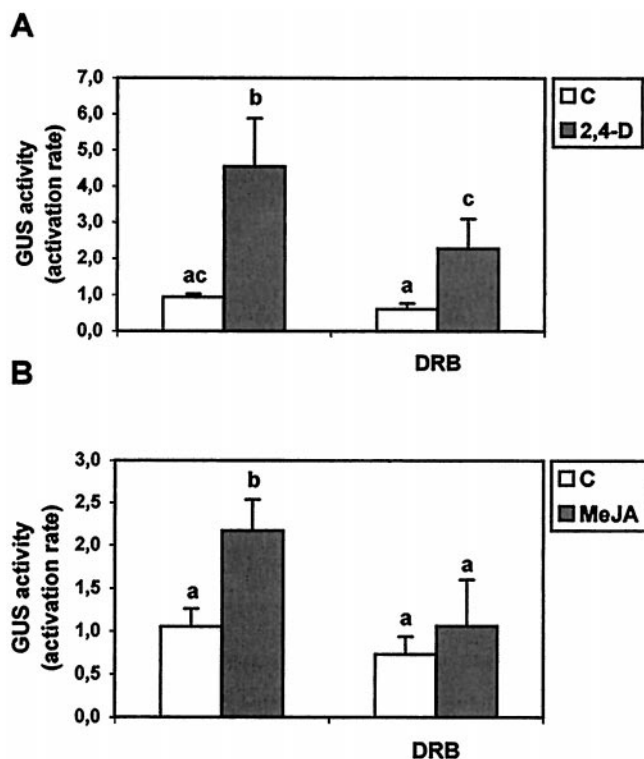


Figure 4. Expression of the (*as-1*)₄/*GUS* gene induced by 2,4-D and MeJA is inhibited by DRB. Leaf discs obtained from tobacco plants transformed with the (*as-1*)₄/*GUS* gene were pretreated for 30 min with 500 μ M DRB or 0.5% (v/v) DMSO and then subjected to the following treatments: A, 100 μ M 2,4-D or 0.5% (v/v) DMSO as a control (C); B, 50 μ M MeJA or 0.5% (v/v) DMSO-1% (v/v) ethanol as a control (C). After 5 h of treatment, GUS activity was measured in protein extracts obtained from these samples. Values are expressed as the activation rate between control and treated samples and represent mean \pm SD of four independent experiments (each done in triplicate). Different letters indicate significantly different values ($P < 0.05$; ANOVA followed by Tukey HSD's test).

vation of genes controlled by the *as-1* cis-element was suggested (Jupin and Chua, 1996; Stange et al., 1997). It is interesting that in this study we also showed that SA produces a significant increase in the nuclear CK2 activity, which gives a stronger support for the participation of this type of PK in the SA-mediated signal transduction pathway.

It is interesting that unlike the activation of late defense genes (e.g. pathogenesis-related (*PR*) genes), activation of early defense genes (e.g. *GST* genes) represents a point of convergency of several signal transduction pathways activated by different hormones, such as SA, MeJA, and auxins. Consistently, functional *as-1*-like promoter elements, which are responsive to these hormonal signals (SA, MeJA, and auxins) have been found in *GST* genes (Ulmasov et al., 1994; Droog et al., 1995; Chen and Singh, 1999). Considering that *GST* proteins play a protective role against the oxidative and chemical stress (Marrs, 1996), activation of their genes may represent a common response triggered by different signals to elicit

protective responses against stress. Unlike *GST* genes, *PR* genes seem to be activated as a more specific response of protection against pathogen-induced stress.

Two main questions remain open concerning to the participation of a CK2 in the mechanism of gene activation by SA. The first one concerns the identification of the target(s) protein(s) for CK2 activity in the SA-activated pathway and, the second one, the mechanism by which SA produces an increase in the nuclear CK2 activity.

Among the possible candidates for target proteins of CK2 activity are the TGA/OBF transcription factors known to bind to the *as-1*-like elements. Four members of this gene family (TGA1a, PG13, TGA2.1, and TGA2.2) have been isolated from tobacco (Kata-giri et al., 1989; Fromm et al., 1991; Niggeweg et al., 2000b). A recent report indicates that TGA2.2 is the main component of the complex that binds to *as-1* sequence in tobacco cellular or nuclear extracts, suggesting an important role for this protein in the response to SA (Niggeweg et al., 2000a). Nevertheless, it is not known whether the responsiveness to SA may be due to phosphorylation of this factor. Biochemical evidence, obtained from in vitro assays of the *as-1* binding activity of TGA/OBF factors present in total or nuclear tobacco extracts, indicate that phosphorylation of nuclear proteins is required for the activating effects of SA on the *as-1* binding activity (Jupin and Chua, 1996; Stange et al., 1997; this study). Furthermore, the observation that dissociating agents increase per se the *as-1* binding activity of total extracts, suggests that phosphorylation may play a role in activating the dissociation of the factor from other proteins leading to the release of the active factor (Jupin and Chua, 1996). The NPR1 protein, which interacts with members of the TGA/OBF family increasing its DNA binding activity (Zhang et al., 1999; Després et al., 2000; Niggeweg et al., 2000b) could be also considered as a putative CK2 substrate. NPR1 has been characterized as an important component of the pathway leading to activate *PR* genes by SA (Cao et al., 1997). Nevertheless, NPR1 seems not to be required to activate *GST* genes by SA or 2,4-D (Uquillas and Holuigue, unpublished data), suggesting that this protein does not play a role in the pathway leading to activate early defense genes by SA. Therefore, whether one of the TGA factors itself or another protein that interacts with it is the CK2 substrate remains to be determined.

Function and Regulation of CK2 Activity

CK2 is a ubiquitous ser/thr PK present in the nucleus and the cytoplasm of eukaryotic cells with an heterotetrameric structure composed by two catalytic (α or α') and two regulatory (β) subunits (Pinna, 1990; Allende and Allende, 1995). At present, more than 160 proteins have been recognized as endoge-

nous substrates for CK2, including enzymes that control DNA and RNA synthesis, transcription and translation factors, and other proteins crucial for cell growth, proliferation, and differentiation (Allende and Allende, 1995). One of the roles generally attributed to CK2 is as a key regulator of the cell cycle (Allende and Allende, 1995). Consistently, increased expression and activity of CK2 has been detected in actively proliferating animal and plant cells (Bosc et al., 1999; Espunya et al., 1999).

Although CK2 was one of the first PKs to be described, it is not clear yet whether its activity in non-dividing cells is constitutive or is subjected to regulation in vivo (Allende and Allende, 1995; Dobrowolska et al., 1999). A transient activation of CK2 (1.3- to 6-fold) was reported to occur in animal cells upon stimulation with polypeptidic hormones like insulin (Sommercorn et al., 1987; Klarlund and Czech, 1988; Kim and Kahn, 1997), insulin-like growth factor (Klarlund and Czech, 1988), and epidermal growth factor (Sommercorn et al., 1987; Ackerman et al., 1990). These results, however, have been challenged on grounds of lack of reproducibility (Litchfield et al., 1991). In this context, our results are of interest as they represent the first evidence for the activation of a potential plant CK2 enzyme by a stress-induced hormone. Further work is certainly needed to elucidate the mechanism by which SA increases the nuclear CK2 activity.

CK2 in Transcriptional Control of Gene Expression

Several authors have reported the participation of CK2 in transcriptional activation of genes, through the phosphorylation of transcription factors (for review, see Allende and Allende, 1995). In some cases (involving c-Jun, serum response factor, Sp1, and Myb) a clear effect of the CK2-mediated phosphorylation on the DNA binding activity of the factor has been established (Lüscher et al., 1990; Manak et al., 1990; Lin et al., 1992; Armstrong et al., 1997).

In plants, the participation of CK2 in gene transcription regulation has been described in three models. In one of these models, CK2 mediated phosphorylation was reported to increase the binding activity of the light-regulated bZIP G-box-binding factor to G-box DNA elements (Klimczak et al., 1995). This CK2 mediated phosphorylation was also demonstrated to be relevant for the in vivo regulation of this factor by light (Harter et al., 1994). More recently, further evidences for the participation of CK2 in light-regulated gene expression were reported using antisense expression of CK2 α gene in Arabidopsis (Lee et al., 1999). In a second model, phosphorylation by CK2 of the bZIP transcription factor Opaque2 involved in maize seed development was reported (Ciceri et al., 1997). This phosphorylation affects its binding to DNA (Ciceri et al., 1997). In a third model, phosphorylation by CK2 of two proteins related to

the control of the circadian rhythms (circadian clock-associated 1, CCA1, and late elongated hypocotyl, LHY, proteins) was reported (Sugano et al., 1999). Further evidences for a role of CK2 in regulation of the circadian rhythms were reported using Arabidopsis plants overexpressing a CK2 β gene (Sugano et al., 1999).

Results reported in this study reinforce a role for CK2 in control of gene expression in plants, in this case in association with defense responses against stress conditions.

MATERIALS AND METHODS

Gene Construct

For construct (*as-1*)₄/*GUS*, the following DNA fragment containing four copies of the *as-1* sequence (DNA binding motifs in bold) was generated by annealing and ligation of four complementary single-stranded oligonucleotides: 5'-agctt(CTGACGTAAGGGATGACGCAC)₂tctaga(CTGACGTAAGGGATGACGCAC)₂c-3' and 3'-a(GACTGCATTCCTACTGCGTG)₂ agatct(GACTGCATTCCTACTGCGTG)₂ gagct-5'.

This fragment was cloned upstream of the truncated -46 to +8 35S CaMV minimal promoter of the X-GUS-46 plasmid (Benfey et al., 1990), using the *Hind*III and *Xho*I cohesive ends designed in the 5'- and 3'-ends of the synthetic DNA fragment (underlined). Correct insertion of this sequence was corroborated by DNA sequencing. The *Hind*III (5')-*Eco*RI (3') fragment containing the tetramer of the *as-1* sequence fused to the -46 truncated 35S promoter, to the *GUS* reporter gene, and to the 3'-polyadenylation sequence from pea *rbc53C* gene, was inserted into the pBI121 binary vector (CLONTECH Laboratories, Palo Alto, CA), in replacement of the CaMV 35S/*GUS*/3'*NOS* cassette. This pBI-derived plasmid containing the chimeric (*as-1*)₄/*GUS* reporter gene (pBI-*as-1*-*GUS* plasmid), was introduced into *Agrobacterium tumefaciens* strain LBA4404 by electroporation (Singh et al., 1993).

Plant Transformation

Tobacco (*Nicotiana tabacum* cv Xanthi nc) plants used for transformation were propagated in vitro in Murashige and Skoog (MS) medium under controlled conditions (20°C–22°C, 16-h light). Transformation was carried out by infection of leaf sections with *A. tumefaciens* strain harboring the binary vector pBI-*as-1*-*GUS*. After infection, transgenic shoots were selected for resistance to kanamycin. Selected shoots were rooted in MS medium supplemented with 50 $\mu\text{g mL}^{-1}$ kanamycin. Independent transgenic clones were cultivated in vitro and then transferred to soil and grown in the greenhouse (15°C–25°C, 16-h light).

Chemical Treatments of Plant Tissues

All the experiments were performed with tobacco plants grown in the greenhouse (15°C–25°C, 16-h light) and containing eight to 12 expanded leaves. To assay *as-1*-binding

activity or CK2 activity, wild-type tobacco (*N. tabacum* cv Xanthi nc) plants were used. To assay transcriptional activity of the (*as-1*)₄/*GUS* reporter gene or the endogenous *gnt35* gene, transgenic tobacco plants harboring the (*as-1*)₄/*GUS* gene were used. For treatments, tobacco leaf discs of 1.2 cm in diameter were punched out and placed in a solution containing 1 mM SA, 100 μM 2,4-D, or 50 μM MeJA. Stock solutions of 2,4-D and MeJA were prepared in DMSO and ethanol, respectively. Final concentrations of DMSO and ethanol in the medium were 0.5% and 1% (v/v), respectively. Control samples were incubated in water (SA) with 0.5% (v/v) DMSO (2,4-D) or 1% (v/v) ethanol (MeJA). Samples were vacuum-infiltrated for 2 min and incubated under constant light in a growth chamber (22°C–25°C) with shaking (50 rpm). After incubation for the periods of time indicated in each case, leaf discs were immediately frozen in liquid nitrogen and stored at -70°C. To assay the in vivo effect of the CK2 inhibitor DRB, leaf discs were first pre-incubated in a solution containing the inhibitor (500 μM DRB) or water in the presence of 0.5% (v/v) DMSO. A stock solution of DRB was prepared in DMSO (final DMSO concentration 0.5% [v/v]). Samples were vacuum-infiltrated and incubated as described above. After 30 min of pre-incubation, SA, 2,4-D, or MeJA was added, samples were vacuum-infiltrated, and incubation was continued for the period of time indicated. After treatments, leaf samples were frozen and stored under the conditions described above.

GUS Activity Assay

To prepare protein extracts, frozen leaf discs were ground in liquid nitrogen; extracted with a buffer containing 50 mM sodium phosphate, pH 7.0, 1 mM EDTA, 0.1% (v/v) Triton X-100, and 10 mM 2-mercaptoethanol; and centrifuged for 10 min at 4°C in a microfuge. GUS activity was assayed by the fluorescent method as described by Jefferson (1987). 4-Methylumbelliferyl glucoside was used as substrate and the fluorescence of the 4-methylumbelliferone (MU) released was measured by emission at 455 nm, after excitation at 365 nm (Fluorometer Hoefer TKO 100, Hoefer Scientific Instruments, San Francisco). GUS activity was expressed as pmol MU min⁻¹ mg⁻¹ protein. A series of MU standard solutions prepared in 0.2 M Na₂CO₃ were used for calibration. Protein concentration was determined using the protein assay (Bio-Rad Laboratories, Hercules, CA).

RNA Extraction and Northern Analysis

Total RNA was extracted from frozen leaf samples as described (Logemann et al., 1987). RNA (20 μg) was resolved on formaldehyde-agarose gels and blotted onto nylon membranes (Hybond N, Amersham, Buckinghamshire, UK). Membranes were hybridized in a buffer containing 6 \times SSC, 5 \times Denhardt's solution, 50% (v/v) formamide, 0.5% (w/v) SDS, 1 mM EDTA, 150 $\mu\text{g mL}^{-1}$ salmon sperm carrier DNA, and ³²P-labeled probe (3–5 \times 10⁷ cpm). Then filters were washed twice in 2 \times SSC, 0.5% (w/v) SDS for 10

min at 55°C. DNA probes of 410 and 130 bp from tobacco *gnt35* and actin genes, respectively, were synthesized by PCR using synthetic primers designed from known sequence data (GenBank accession nos. X56269 for pGNT35 and X63603 for NTACTION). These probes were labeled with [α - 32 P]dCTP by random oligonucleotide-primed synthesis (Megaprime DNA labeling system, Amersham).

Nuclear Extracts

Nuclear extracts were prepared from leaves of treated plants according to the procedure described by Green et al. (1989) and modified as follows. Frozen tobacco leaf samples (approximately 4 g of fresh weight) were ground in a mortar with liquid nitrogen and resuspended in 25 mL of homogenization buffer (1 M hexylene glycol, 10 mM PIPES [1,4-piperazinediethanesulfonic acid]/KOH, pH 7.0, 10 mM MgCl₂, 10 mM NaF, 10 mM KCl, 1 mM EDTA, 0.5% [v/v] Triton X-100, 1 mM 2-mercaptoethanol, 0.8 mM phenylmethylsulfonyl fluoride). The homogenate was filtered through two layers of Miracloth and nuclei were sedimented at 3,000g for 2 min. The pellet was resuspended in 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 phenylmethylsulfonyl fluoride) with 0.5% (v/v) Triton X-100, and nuclei were sedimented at 3,000g for 1.45 min. The nuclear pellet was resuspended in 10 mL of nuclei wash buffer, sedimented at 3,000g for 1.45 min, and resuspended in 5 mL of lysis buffer (15 mM HEPES/KOH, pH 7.5, 110 mM KCl, 5 mM MgCl₂, 10 mM NaF, 100 μ M EDTA, and 2 μ g mL⁻¹ of leupeptin, pepstatin A, and aprotinin). After 1 h on ice, ammonium sulfate was added and fractionation was performed as described (Green et al., 1989). After the second fractionation, the pellet was resuspended in 100 μ L of nuclear extract buffer (25 mM HEPES/KOH, pH 7.5, 40 mM KCl, 100 μ M EDTA, 15% [v/v] glycerol, 10 mM NaF, and 2 μ g mL⁻¹ of leupeptin, pepstatin A, and aprotinin), dialyzed for 3.5 h against the same buffer and stored at -70°C. Protein concentration was determined using the Bio-Rad Laboratories kit.

Gel Mobility Shift Assays

To obtain the *as-1* element DNA probe for protein binding assays, the oligonucleotide 5'-CTGCAGACTGACGT-AAGGGATGACGCACAACCTCGAG-3' was used (protein binding motifs are indicated in bold). The complementary strand was synthesized and labeled using the primer 5'-CTCGAGT-3', [α - 32 P]dCTP, dNTPs, and Klenow DNA polymerase, following standard protocols (Sambrook et al., 1989). DNA-protein binding assays were performed in 12 μ L of a medium containing 50 mM HEPES, pH 7.9, 100 mM KCl, 2 mM MgCl₂, 20 mM dithiothreitol, 3.75% (v/v) glycerol, 10 mM NaF, 8 mM Na₂MoO₄, and 15 ng poly(dG)·poly(dC) (Pharmacia Biotech, Piscataway, NJ), radioactively labeled probe (25,000 cpm, 0.12 pmol), and nuclear protein extract (5 μ g of protein). After 20-min incubation at room temperature, DNA-protein complexes were sepa-

rated from the unbound probe by electrophoresis in a 6.072% T (1.186% C) polyacrylamide gel in Tris-borate/EDTA. After electrophoresis, gels were dried and subjected to autoradiography at -70°C with enhancer screens for 16 h.

CK2 Activity Assay

CK2 activity was assayed in nuclear extracts obtained from tobacco leaves subjected to the indicated treatments. CK2 assays were performed in 30 μ L of medium containing 50 mM HEPES/KOH, pH 7.9, 8 mM MgCl₂, 10 mM KCl, 10 mM NaF, 1 mM peptide RRRDDDSDDD, 200 μ M [γ - 32 P]ATP or [γ - 32 P]GTP (400–500 cpm pmol⁻¹), and nuclear extract (5 μ g of protein). To assay the effect of heparin or DRB, these compounds were added directly to the reaction medium. After incubation for 15 min at 30°C, reaction mixtures were spotted onto Whatman P81 ion-exchange papers. Papers were washed three times with 75 mM phosphoric acid, dried, and radioactivity was quantitated with a liquid scintillation counter. To determine the specific 32 P-incorporation into the substrate peptide, the incorporation of label into endogenous nuclear proteins obtained in the absence of the peptide was subtracted from the total 32 P-incorporation. CK2 specific activity was expressed as pmol 32 P incorporated into the peptide min⁻¹ mg⁻¹ protein. Activity was linear with the amount of extract assayed in the range of 2.5 to 10 μ g of protein in 30 μ L of reaction medium.

Statistical Analysis of Data

Differences in the CK2 activity between control and SA-treated samples (Fig. 1) were evaluated by the Student's *t* test. Differences in the GUS activity among treatments (Figs. 3A and 4) were tested by one-way ANOVA and Post Hoc Tukey HSD's Multiple-Comparison Test, using the statistical program SPSS 7.5 (SPSS, Inc., Chicago).

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