Identification of Methyl Jasmonate and Salicylic Acid Response Elements from the Nopaline Synthase (nos) Promoter

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Transgenic tobacco plants carrying a fusion between the nopaline synthase (nos) promoter and chloramphenicol acetyltransferase (CAT) reporter gene (cat) were studied for their inducibility by salicylic acid (SA) or methyl jasmonate (MJ) treatments. Either chemical significantly increased CAT activity to a level much higher than that achieved by wounding. Northern blot analysis showed a corresponding increase in mRNA levels. After 20 h of induction of flowering plants, the response to MJ treatment was weaker in old leaves compared with young leaves, whereas the SA response was stronger in old leaves. Kinetic experiments showed that the SA response was much faster than the MJ response, suggesting that the induction mechanism of the nos promoter by these chemicals may differ. Deletion analysis showed that both SA and MJ responses require the DNA sequence between -119 and -112 from the transcription initiation site. This region contains the hexamer sequence (TGACGT) that has been found to be an important regulatory element for several promoters. The MJ response was also reduced by deletions of the CAAT box region or the sequence between -112 and -101, whereas the SA response was not significantly affected by these deletions. This suggests that the nos upstream region containing the hexamer motif is essential for the SA or MJ response and that the CAAT box region and the sequence immediately downstream from the hexamer motif are required for maximum induction by MJ.

The soil bacterium Agrobacterium tumefaciens induces a crown gall disease on wound sites of most dicotyledonous and some gymnosperm plants (Nester and Kosuge, 1981). The large hypertrophies resulting from autonomous cell proliferation are associated with stable covalent integration of a part of the tumor-inducing plasmid into the plant chromosome. Several genes present in the T-DNA are actively transcribed in transformed tumor tissues (Drummond et al., 1977). One of the most abundant transcripts in the nopaline-type tumor tissues is that of the nopaline synthase gene (nos). The nos promoter has been used for construction of plant selectable markers (Lichtenstein and Fuller, 1987), because the nos gene was considered to be constitutively active in various plant tissues. However, it was later shown that the nos promoter activity is very weak in transgenic tobacco plants and varies significantly among different organs and developmental stages (An et al., 1988a). Because the nos promoter is strongly active in tumor tissues, we have searched for positive regulatory factors that are present in tumor tissues. Tumor formation by Agrobacterium is due to wound response and a high level of auxin and cytokinin produced from T-DNA genes. It was found that nos promoter activity is inducible by wounding and further enhanced by auxin in both vegetative and reproductive organs (An et al., 1990). Other phytohormones such as BA, ABA, and GA had no significant effect. In this study, we report that the nos promoter is also inducible by MJ and SA.

 Jasmonic acid and its methyl ester are widely distributed in the plant kingdom (Yamane et al., 1981; Meyer et al., 1984). It was previously observed that direct application of jasmonate or MJ inhibited growth and promoted senescence and abscission (Ueda and Kato, 1980; Weidhase et al., 1987; Staswick et al., 1992). Among MJ-inducible genes are proteinase inhibitor genes of tomato and potato (Farmer and Ryan, 1990; Kim et al., 1992a) and soybean leaf vegetative storage protein genes (Franceschi and Grimes, 1991), which are also wound inducible. Jasmonate and MJ accumulate rapidly and transiently after wounding (Creelman et al., 1992) or treatment of plant suspension cultures with a yeast elicitor (Gundlach et al., 1992). Addition of MJ to suspension culture initiates de novo transcription of genes such as Phe ammonia lyase that are known to be involved in the chemical defense mechanisms of plants. This suggests that MJ may be an important stress-signaling molecule in plants. It has been proposed that wounding triggers biosynthesis of jasmonic acid and MJ from linolenic acid (Farmer and Ryan, 1992).

SA has been recognized as an endogenous regulatory signal in plants, especially during plant defense against pathogens (Malamy et al., 1990; Metraux et al., 1990; Raskin, 1992). Systemic induction of PR proteins in tobacco, which occurs during the hypersensitive response to TMV, is accompanied by at least 10-fold systemic increase of SA in TMV-resistant, but not in TMV-susceptible, tobacco (Yalpani et al., 1991). It has been postulated that SA is accumulated to serve as the systemic signal molecule that induces synthesis of PR proteins and other compounds in resistant plants. Exogenous appli-

Abbreviations: CAT, chloramphenicol acetyltransferase; MJ, methyl jasmonate; MS, Murashige and Skoog; PI, proteinase inhibitor; PR, pathogenesis related; SA, salicylic acid; T-DNA, transfer DNA; TMV, tobacco mosaic virus.

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cation of SA or its derivative acetyl SA induced accumulation of PR proteins and resulted in an increased resistance of the treated areas to virus and fungi (Ward et al., 1991; Enyedi et al., 1992; Uknes et al., 1992). Because the nos promoter is inducible by SA and MJ, we have identified the regulatory regions that are necessary for the response to these stimuli.

**MATERIALS AND METHODS**

**Generation of the CAAT Box Deletion Mutants**

The plasmid containing the nos 5’ deletion mutant 5’-92 (An et al., 1986) was opened at the unique BglII site, which is located at the deletion point, -92. After BAL-31 and Klenow polymerase treatments, the mutated promoter fragments were linked to the 3’ deletion mutant 3’-97 (An et al., 1986), generating internal deletions that start at -97 and end at a different position of the nos promoter. The deletion end points were determined by the Maxam and Gilbert (1977) DNA-sequencing method. The mutant promoters were placed upstream of the cit reporter gene and subcloned into a binary tumor-inducing plasmid vector, pGA628 (An et al., 1988b). The plasmids were transferred into Agrobacterium tumefaciens by the direct DNA transformation method and isolated by the alkaline lysis method for verification of the structure (An et al., 1988b).

**Plant Materials and Transformation**

Transgenic plants were obtained by the cocultivation method (An et al., 1988b) using tobacco (Nicotiana tabacum L. cv Petit Havana SR1) plants grown aseptically on MS agar medium supplemented with 3% Suc (Murashige and Skoog, 1962). Kanamycin-resistant transformants were selected and grown in the greenhouse, and seeds were collected after self-fertilization. Seeds were germinated on MS agar medium containing 50 μg mL⁻¹ of kanamycin, and plants were grown either in a Magenta square box or under greenhouse conditions.

**MJ and SA Induction Assays**

All of the experiments were performed with the first or second progenies of transgenic plants. Leaves were wounded by cutting into pieces, which were floated on MS liquid medium for 20 h at room temperature. MJ (Bedoukian Research Inc., Danbury, CT) or SA (Sigma) was included in MS medium during the incubation period. The CAT assay was conducted using 0.4 to 100 μg of total soluble protein at 37°C for 30 min by a TLC method using [¹⁴C]chloramphenicol as described previously (An et al., 1988b).

**Northern Blot Analysis**

Mature leaves from the greenhouse-grown plants were harvested and wounded as described above. Total RNA (50 μg), which was isolated by the guanidinium thiocyanate method, was separated on a formaldehyde gel, blotted onto a nylon membrane, and hybridized with radioactively labeled probe prepared from the cat-coding region as described previously (Sambrook et al., 1989).

**RESULTS**

**SA and MJ Effects on the nos Promoter**

Because SA and MJ are considered to be signal molecules that induce a variety of plant genes involved in wound or defense responses, we examined whether these chemicals could also affect the nos promoter activity. Leaves of transgenic tobacco plants carrying the fusion molecule between the nos promoter and cat reporter genes were treated with SA or MJ for 20 h. The results shown in Figure 1 indicate that 0.1 mM SA or 10 μM MJ significantly induced the nos promoter activity in leaves of transgenic plants. Higher concentrations of these chemicals further increased promoter activity. Stems and petioles also respond to the chemical treatments (data not shown). At the flowering stage, the nos promoter activity induced by SA treatment was up to 40% higher in old leaves, whereas the response to MJ was about 2-fold higher in young leaves. When plants matured, the basal level of nos promoter activity increased, whereas the MJ response was reduced in all leaves (data not shown).

Because the nos promoter is also wound and auxin inducible (An et al., 1990), we compared the SA or MJ inducibility with that of wounding and 2,4-D (Fig. 2). The levels induced by MJ or SA were not as high as that induced by 2,4-D but were still significantly greater than the wound-induced level. Addition of SA and MJ simultaneously did not produce any additive effect. Samples from old leaves at the flowering stage were used for northern blot analysis (Fig. 3). This

![Figure 1](image-url)
Methyl Jasmonate and Salicylic Acid Response Element of nos Promoter

Figure 2. Comparison of Mj and SA responses with the 2,4-D response. Leaf segments were sampled before (C) or after 20 h of treatment with wounding (W), 0.2 mM SA (S), 10 μM Mj (M), 0.9 μM 2,4-D (A), or 0.2 mM SA plus 10 μM Mj (SM). The CAT activity was measured using 4 μg of total soluble protein for the nos and cab-nos hybrid promoters or 0.4 μg for the 35S promoter. Average values of four independent transgenic plants. Bars, SD.

Figure 3. Northern blot analysis. Old leaves at flowering stage were treated as described in Figure 2. Total RNAs (50 μg) were separated on a denaturing agarose gel, blotted onto a nylon membrane, and hybridized with the radioactively labeled probe prepared from the cat-coding region. Numbers on the right indicate RNA size markers.

showed that SA or Mj treatment increased the mRNA level of the cat reporter gene, supporting the CAT enzyme assay data (Fig. 2) and verifying that the induction occurred at the mRNA level. To investigate whether the induction was due to increased nos promoter activity or cat mRNA stability, transgenic tobacco plants carrying the 35S promoter-cat fusion were studied (Fig. 2). If the SA or Mj treatment increases mRNA stability, higher CAT activity is expected from these 35S promoter-cat transgenic plants. Wounding leaves of the plants slightly induced CAT activity as observed previously (Kim et al., 1991). However, SA or Mj did not increase the CAT activity above the wound-induced level in 35S promoter-cat plants. This indicates that SA or Mj treatment does not increase cat mRNA stability but that it induces the nos promoter activity.

Kinetic studies were carried out to investigate the rate of induction by Mj, SA, and 2,4-D. As shown in Figure 4, the CAT activities started to increase by 30 min and continued to increase during longer incubation. This experiment also revealed that the SA response was induced more rapidly than the response to Mj. During the first 10-h incubation period, the SA-induced levels were significantly higher than those induced by Mj. However, after 20 h of incubation, the Mj-induced level approached that of the SA treatment. On the
other hand, the 2,4-D treatment showed a significantly different kinetic response. During the first 4 h, the 2,4-D response was slower than the SA response. However, after 6 h of treatment, the 2,4-D response surpassed the levels obtained by SA or MJ treatment. The wound induction curve was similar to that of MJ but at a reduced level. This supports the model that MJ is the wound factor.

Identification of the SA and MJ Response Regulatory Region

To define the cis-acting regulatory region responsible for the SA and MJ induction, the CAT activity response to these chemical treatments was studied in transgenic tobacco plants carrying various deletion mutants of the nos promoter fused to the cat reporter gene and the terminator region of a T-DNA gene 6b. For each deletion mutant, we tested at least four transgenic plants that previously showed positive expression of the cat reporter gene (Ha and An, 1989). Leaves from greenhouse-grown young plants were induced with SA or MJ. Although there was variation in the CAT activity among independent transgenic plants, the induction pattern was identical for the transgenic plants containing the same deletion mutant.

Figure 5 shows the CAT assay data for a representative sample from each mutant (left) and the relative CAT activity of control, wound, and MJ treatments normalized to the level induced by SA (right). These data show that the full-length nos promoter (5'-263) and the 5' deletion mutant -155 were induced to almost equal levels by either SA or MJ. The deletion mutant 5'-130 was also inducible by both stimuli, although the level of induction was not as strong as in the full-length nos promoter or the 5' deletion mutant -155. These experiments indicated that the regulatory element inducible by SA or MJ is present downstream of -130 and that the region between -130 and -155 positively modulates the promoter activity.

We showed previously that the cis-acting regulatory element essential for the wound or auxin response is present at the region between -130 and -101 (An et al., 1990). To study whether this region is also essential for the SA or MJ responses, internal deletion mutants lacking this upstream region were studied. As shown in Figure 5, the mutant -119/101 lost the response to SA or MJ, whereas the mutant -112/101 retained the response. However, in the mutant -112/101, the MJ response was not as strong as the SA response. These results suggest that the sequence between -119 and -112 is essential for both SA and MJ responses and that the sequence between -112 and -101 enhances induction by MJ. These experiments do not resolve whether this region indeed carries a regulatory sequence responsive to these stimuli or whether it contains a constitutive enhancer element that enhances an MJ- or SA-inducible element located at the downstream region.

To investigate the role of the downstream region of the nos promoter, transgenic plants carrying a fusion between the upstream promoter region of an Arabidopsis Chl a/b-binding protein gene (cab) and the truncated nos promoter 5' deletion mutant -101 were studied (Ha and An, 1989). If the nos promoter sequence downstream of -101 contains an SA-MJ-inducible element that is not functional by itself but requires an enhancer sequence for promoter activity, the cab-nos fusion promoter should be inducible by the chemical treatments. However, as shown in Figure 2D, this promoter is not inducible by SA or MJ. Therefore, it can be concluded that the nos promoter sequence upstream of -101 is required for the induction.

To study further the role of the CAAT and TATA box sequences, transgenic plants carrying deletions of the region...
were also studied. The 3' deletion mutant (3'-25), which lacks the TATA box region, responded to MJ and SA, although the induced levels were much weaker. This indicates that the TATA box is not required for the chemical responses. The CAAT box deletion mutant (−81/63) also responded at a reduced level to both chemicals. However, as observed from the mutant −112/101, the MJ response was much weaker than the response to SA treatment. Therefore, it seems that the CAAT box region is also necessary for the maximum response to MJ.

CAAT Box Deletion Mutants

Because the CAAT box region appears to be involved in the MJ response, we generated internal deletion mutants using exonuclease BAL-31 as described in “Materials and Methods.” Transgenic tobacco plants carrying these deletion mutants were analyzed for inducibility by MJ and SA (Fig. 6). The deletion between −97 and −83 did not affect inducibility by either chemical. The levels induced by SA and MJ were almost identical with each other, as was observed with the wild-type promoter (Fig. 5, 5'-263). However, deletion of four nucleotides between −83 and −79 significantly reduced the MJ response compared to the response of SA. Further deletions to −75, −70, or −68 showed similar responses, indicating that the regulatory element involved in the MJ response is located downstream of −83, where the CAAT box sequence is located.

DISCUSSION

We have shown in this study that the nos promoter is inducible by SA and MJ, which are considered to be important signal molecules during plant defense and wound response, respectively. These results, together with the previous observation that the promoter is wound and auxin inducible, suggest that the nos gene is actively transcribed in tumor tissues, probably because of the presence of high levels of these signals. The gene is weakly active in young leaves in which there may be lower amounts of these signals. The observation that the basal level of the nos promoter is higher in older leaves suggests that the endogenous level of at least one of these chemicals increases in older tissues.

The MJ inducibility was much weaker in old leaves, in contrast to the SA response, which was greater in old leaves. One possible reason for this difference is that there might be an inhibitor of the MJ response accumulating in older leaf tissues. Alternatively, the level of MJ receptor may decrease when cells get older, or it may be more difficult for the MJ signal to travel through older tissues. The induction kinetics experiments showed that the SA response was much faster compared to the MJ response. The SA response began to level off by 10 h of incubation, whereas the 2,4-D response continued to increase up to 20 h, at which time the difference between the two treatments was approximately 6-fold. This indicates that the SA induction mechanism is probably different from that of MJ or auxin.

Although the nos promoter is similar to PI promoters with respect to wound and MJ responses, it remains unresolved whether the induction mechanism is similar. The observation that the PI and vegetative storage protein genes are strongly induced in old leaves suggests that the MJ induction of the nos promoter may differ from that of PI or vegetative storage protein promoters. The potato PI-II promoter, unlike the nos promoter, is not inducible by SA (Kim et al., 1992b) or auxin (An et al., 1990). Also, the PI promoter is inducible by various sugars that do not noticeably affect the nos promoter activity (Johnson and Ryan, 1990; Kim et al., 1991). These results further support the hypothesis that regulation of the nos promoter activity might be different from that of PI promoter.

It was reported that TMV infection increases the endogenous level of free SA up to about 0.1 μM, causing induction of various PR protein genes (Enyedi et al., 1992; Malamy et al., 1992). A much higher concentration of conjugated SA is further support the hypothesis that regulation of the nos promoter may differ from that of PI or vegetative storage protein genes (Johnson and Ryan, 1990; Kim et al., 1991). These results further support the hypothesis that regulation of the nos promoter activity might be different from that of PI promoter.

Figure 6. Deletion analysis of the CAAT box region. Leaves of transgenic plants carrying various CAAT box deletion mutants were treated as described in Figure 2. Results from a representative sample are shown in TLC autoradiographs on the left. Relative CAT activities of control (C), wound (W), and MJ (M) treatments normalized to the 100% base level induced by SA (S) are shown on the right. The data consist of average values of two to four independently transformed plants. The amount of total soluble protein used for the CAT assay was 100 μg. Bars, so.
occurring in natural conditions. A toxic effect on incubated leaves was observed only when the SA level was greater than 2 mM. Therefore, induction of the nos promoter by much lower amounts of exogenously applied SA is unlikely due to an indirect side effect such as leaf senescence. It was observed from soybean suspension cultures that lower concentrations (1–10 μM) of MJ or jasmonic acid alter protein and mRNA populations, whereas high concentrations (greater than 30 μM) induce senescence (Anderson, 1988). In this study, we have shown that the nos promoter activity is significantly inducible by 10 μM MJ, and higher concentrations moderately enhance the effect. We did not observe any noticeable change in Chl or protein content of the leaf tissues treated with 0.2 mM MJ for 20 h.

It was revealed in this study that the nos upstream region between –119 and –112 is essential for the SA and MJ responses. It was demonstrated previously that this region is also essential for wound response and auxin inducibility. Site-specific mutations and fine deletion analysis of the nos upstream region showed that a 20-nucleotide regulatory element located between –131 and –112 is essential for the promoter activity (Y. Kim and G. An, unpublished results). The element consists of two hexamer motifs that are separated by an eight-nucleotide spacer sequence. Because one of the hexamer motifs (TGACGT) is present between –119 and –112, this suggests that this element plays an important role in the MJ and SA responses. DNA sequences similar to the nos hexamer motif region were also found in other promoters such as the ncs (Bouchez et al., 1989; Singh et al., 1989) and 35S (Benfry and Chua, 1990) promoters. These elements were shown to be essential for the activity of these promoters (Lam et al., 1989; Singh et al., 1990). These sequences are specific binding sites for a family of trans-acting factors that are Leu-zipper proteins (Katagiri et al., 1989; Tabata et al., 1989; Singh et al., 1990). Whether SA or MJ treatment induces the nos promoter activity by increasing the level of the transcription factor and/or by activating the protein remains to be determined. The cDNA clones for the trans-acting factors will be useful in solving these problems.

We have shown earlier that the G-box sequence (TCACGTGG) of the potato PI-II promoter is essential for the MJ response (Kim et al., 1992a). Because the first six nucleotides of this element and the nos hexamer motif are the same except for the second nucleotide, it is possible that there is a common or similar transcription factor inducible by MJ that interacts with these regulatory sequences. This is in agreement with the report that a transcription factor GBF-1 of Arabidopsis thaliana interacts with both G-box and hexamer sequences (Schindler et al., 1992b). However, it should be noted that the sequence immediately downstream of the nos hexamer sequence is AA, whereas it is GG in the PI-II promoter. Because it appears that the binding specificity of certain trans-acting factors is determined by the flanking sequence (Schindler et al., 1992a; Williams et al., 1992) and the G-box element is essential for an ABA response (Guiltrain et al., 1990), it is likely that the nos hexamer and G-box regions are regulated by different trans-acting factors. It is unknown whether wounding, MJ, SA, and auxin induce the promoter via a common receptor or whether each treatment is mediated by a different receptor that independently activates the nos promoter. We are currently investigating the molecular mechanisms of the promoter activation by these various stimuli using transcription factors that specifically interact with the nos upstream region.

Deletion of the immediate downstream region of the 20-nucleotide sequence between –112 and –101 significantly reduced the MJ and wound responses as compared to the SA response. Similarly, the CAAT box deletions also resulted in a significant decrease in the MJ and wound responses. However, in these mutants, SA signaling was not affected as much as that of MJ or wounding. That the extent of the wound response in different mutants follows the MJ response rather than the SA response suggests that MJ is a likely wound signal molecule. It can be postulated that the SA response is primarily determined by the transcription factors that interact with the hexamer motif area, whereas for maximum induction, the MJ and wound effect requires additional transcription factors that interact with either the CAAT box or the region between –112 and –101. Deletion analysis of the CAAT box region indicates that the sequence between –97 and –83 is not involved in the MJ response, whereas the sequence downstream from –83 is important. Therefore, it is likely that the CAAT box, which is located in this region, plays an important role in the MJ effect. However, deletion of the entire CAAT box region (~97/68) or the region between –112 and –101 did not completely abolish the response to MJ. This indicates that the upstream hexamer region and the downstream sequence elements function together for the MJ response. Such a synergistic effect between the CAAT box and upstream region was also reported for the soybean heat-shock promoter (Rieping and Schoffl, 1992). A transcription factor that specifically interacts with the CAAT box region is being studied to allow further understanding of the role of the CAAT box region.

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