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Nopaline Synthase Promoter Is Wound Inducible and Auxin Inducible

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The activity of the nopaline synthase (*nos*) promoter is differentially regulated in several plant organs. In this article we demonstrate that the *nos* promoter is wound inducible in both vegetative and reproductive organs. The induction of the *nos* promoter was observed in leaves, stems, cotyledons, and various reproductive organs, suggesting that the response is not organ specific. The wound response was further enhanced by addition of auxins. Other growth substances had no effect on the wound-inducible *nos* promoter activity. Deletion analysis of the *nos* promoter indicated that the 10-base pair (GCACATACGT) *Z* element located between −123 and −114 or an element overlapping with this sequence is essential for the wound and auxin responses.

INTRODUCTION

The transferred DNA (T-DNA) genes of *Agrobacterium* tumor-inducing (Ti) plasmid are actively transcribed in transformed plant cells (Drummond et al., 1977). The flanking regions of T-DNA genes carry typical eukaryotic regulatory sequences such as the TATA box, CAAT box, and polyadenylation signal sequences (Barker et al., 1983). Observations that the T-DNA transcripts are polyadenylated and that the transcription is inhibited by α-amanitin in plant cells (Willmitzer, Schmalenbach, and Schell, 1981; Gelvin et al., 1982) indicate that the regulatory regions of the T-DNA genes interact with host regulatory systems to express the genes in plant cells.

The control regions of several T-DNA genes have been studied. The octopine synthase (*ocs*) upstream region that is necessary for the gene expression in transformed tobacco calli is located between 222 and 177 bp upstream from the transcription initiation site (Leisner and Gelvin, 1988, 1989). This region contains a 16-bp palindrome that is essential and sufficient for activating a corn *Adh1* promoter in tobacco cells (Ellis et al., 1987) and interacts with a nuclear protein factor (Fromm, Katagiri, and Chua, 1989; Singh et al., 1989). Promoters of other T-DNA genes, such as the 780 gene (Bruce and Gurley, 1987; Bruce, Bandyopadhyay, and Gurley, 1988), agropine biosynthase gene (Bruce et al., 1988), mannopine synthase gene (DiRita and Gelvin, 1987), and isopentenyl transferase gene (de Pater et al., 1987), have also been analyzed.

We have been studying the regulatory regions of the nopaline synthase (*nos*) gene, whose product catalyzes the condensation of α-ketoglutarate with arginine to form nopaline. This compound can be metabolized only by *Agrobacterium* as a source of nitrogen and carbon (Nester and Kosuge, 1981). 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 recently observed that the *nos* promoter activity is organ specific and developmentally regulated (An et al., 1988).

We have previously identified at least three regulatory regions from the *nos* promoter (An et al., 1986b; Ebert, Ha, and An, 1987; Ha and An, 1989). The TATA box is required for promoter efficiency in plant cells. Deletion of this region resulted in reduction of promoter activity by about 10-fold. The second element is the CAAT box region, which is essential for *nos* promoter activity in differentiated tissues. However, the CAAT box deletion mutants displayed a significant promoter activity (5% to 20% of the wild type) in cultured cells, suggesting that the requirement for the CAAT box region is more stringent in differentiated cells. The region immediately upstream from the CAAT box is essential for the *nos* promoter to function. Deletion of this region abolished the promoter activity in calli as well as in differentiated tissues. We have observed that this region is also responsible for the vertical gradient of activity in vegetative organs (Ha and An, 1989) and is composed of a 10-bp potential Z-DNA-forming (*Z*) element and at least two additional positive regulators: one at the immediately downstream region and the other at the upstream region from the *Z* element (Mitra and An, 1989). In the present study, we have demonstrated that the *nos* promoter is inducible by mechanical wounding and the wound response is further enhanced by the phytohormone auxin. We have also found that one of the regulatory elements

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involved in the wound response and hormone response of the promoter is the *Z* element or an element which overlaps with this sequence.

RESULTS

Wound Response of the *nos* Promoter

Expression of the *nos* promoter is differentially regulated in a variety of plant organs. To identify the factors that may contribute to this complex pattern of the *nos* promoter activity, leaves of transgenic tobacco plants carrying the *nos* promoter-chloramphenicol acetyltransferase (*cat*) fusion gene (pGA658) (An et al., 1988) were mechanically wounded by cutting into slices and incubated in MS medium. CAT mRNA (Figure 1) and enzyme activity (Figure 2A) derived from the *nos* promoter were significantly induced by the treatment. It was previously observed that promoter activity of the wound-responsive potato proteinase inhibitor II (PI-II) gene was also induced by the same assay (Figure 2B), whereas the activity of the cauliflower mosaic virus (CaMV) 35S promoter was not changed by wounding leaves of transgenic tobacco plants carrying the 35S promoter and *cat* fusion (Figure 2C) (An et al., 1989). More than 50 transgenic tobacco plants carrying the *nos* promoter-*cat* fusion molecule were examined for their inducibility of the promoter. Although only three samples are shown in Figure 2, the *nos* promoter was inducible in every plant, demonstrating that the wound response of the *nos* promoter is independent of position where the promoter is located in the chromosomal genome. As observed earlier, there is a significant variation in the level of gene expression among independent transgenic plants (An, 1986).

To investigate whether a component of the medium used for the leaf slice assay is responsible for the induction of the *nos* promoter, leaf slices were incubated in sterile water. Results in Figure 3A show that the CAT activity was slightly stronger in the Murashige and Skoog (MS) medium (sample d) compared with sterile water (sample c). Wounding leaves in intact plants (sample b) also induced *nos* promoter activity to the level equivalent to that obtained by incubating leaf slices in sterile water. As a control, transgenic tobacco leaves carrying the 35S promoter-*cat* fusion were examined by the same wound treatments. Results in Figure 3B show that wounded leaves did not show any significant change in the 35S-driven CAT activity.

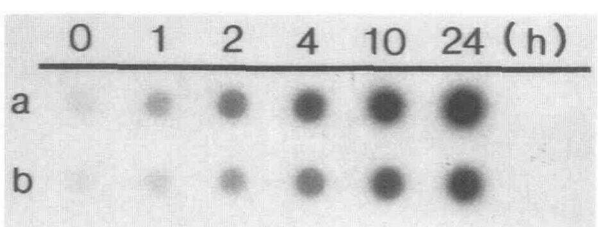

Figure 1. Wound Induction of the *nos* Promoter.

RNA was prepared from tobacco plants carrying pGA658 that were grown to the flowering stage in the greenhouse.
(a) Leaf slices were incubated for 0 hr to 24 hr, and 8 μg of total RNA was spotted on a Zeta-Probe membrane and hybridized with a ^{32}P probe containing the *cat* gene.
(b) Leaf slices were incubated for 0 hr to 24 hr, and 4 μg of total RNA was spotted on a Zeta-Probe membrane and hybridized with a ^{32}P probe containing the *cat* gene.

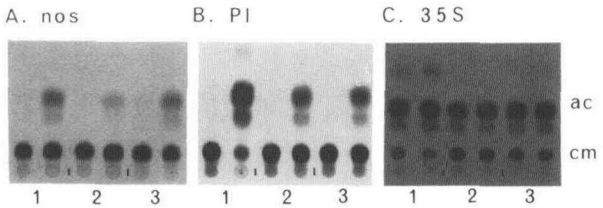

Figure 2. Wound Induction of *nos*, Potato PI, and CaMV 35S Promoters.

(A) Transgenic tobacco plants carrying the *nos-cat* fusion gene were grown in the greenhouse to the flowering stage.
(B) Transgenic tobacco plants carrying the PI-*cat* fusion gene were grown in the greenhouse to the flowering stage.
(C) Transgenic tobacco plants carrying the CaMV 35S-*cat* fusion gene were grown in the greenhouse to the flowering stage.
Results from three plants are shown for each promoter. For each plant, a pool of slices from five young leaves was sampled before (left sample of each pair) and after (right sample of each pair) incubation for 24 hr in MS medium. Numbers indicate independently transformed tobacco plants. Using 4 μg of total soluble protein to standardize samples, CAT activity was assayed by measuring conversion of chloramphenicol (cm) to acetylchloramphenicol (ac).

Wound Response of Various Organs

It has been previously shown that strength of the *nos* promoter is dependent on the locations of individual vegetative organs. The promoter activity is greater in lower parts and the overall expression level is decreased significantly at the flowering stage (An et al., 1988). To investigate whether the wound induction is position dependent and organ specific, cotyledons, leaves, and stems from 11-leaf-stage plants were incubated on MS medium for 24 hr. Results in Figure 4 demonstrate that all of the aboveground vegetative organs are wound inducible and that the wound-induced level is greater in the lower parts of the plant. Similar results were obtained from all of the transgenic tobacco plants tested (Table 1).

To investigate whether the induction is tissue-type specific, mesophyll, epidermal, and midvein tissues were

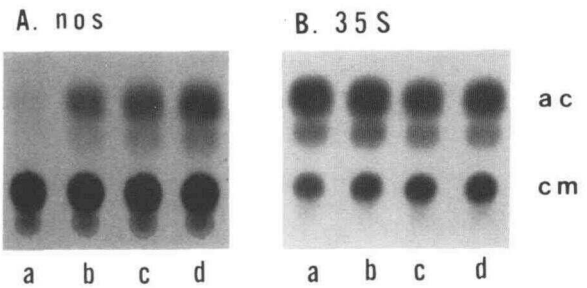

Figure 3. Effect of Wound Conditions on Promoter Activity.

(A) *nos* promoter.
(B) 35S promoter.
Leaves from five independently transformed plants were wounded and incubated using three different treatments. Treated leaves were pooled and CAT activity was measured using samples standardized to 4 μg of total soluble protein. Sample a, unwounded leaves. Sample b, wounded by gently rubbing fingers over surface of intact leaves. After wounding, leaves were left on plants for 24 hr before sampling. Sample c, wounded by cutting up leaves into small sections (approximately 0.5 cm^2) and incubated in sterile water for 24 hr. Sample d, wounded by cutting up leaves into small sections and incubated in MS medium for 24 hr. ac, acetylchloramphenicol; cm, chloramphenicol.

isolated and independently incubated in MS medium. It was observed that all three different leaf tissues were inducible by the mechanical wound treatment (Figure 5), although the mesophyll tissue showed the strongest wound response.

We have previously reported that the *nos* promoter is active in reproductive organs (An et al., 1988). The results in Figure 6 and Table 2 show that the promoter is also wound-inducible in various reproductive organs of all the transgenic tobacco plants tested.

Effects of Plant Growth Substances

The T-DNA genes are actively transcribed in tumor tissues where phytohormones, especially auxin and cytokinin, are abundant (Nester and Kosuge, 1981). Therefore, we have tested whether the wound response is influenced by a phytohormone. Among the various growth substances tested, 2,4-D had the most significant effect on *nos* promoter activity. Addition of 0.2 mg/L 2,4-D to the incubation media enhanced the promoter activity by about twofold compared with the control leaf slices (Figure 7). The effect was more pronounced with higher levels of the growth substance. Naphthaleneacetic acid, indole-3-acetic acid, and indolebutyric acid also increased *nos* promoter activity, but the effects were not as significant as that of 2,4-D. No significant effects were observed with benzylaminopurine, kinetin, abscisic acid, or gibberellic acid (GA_3) (data not shown). The auxin effects appear to be generalized to the whole plant because auxin also enhanced the promoter activity in various reproductive organs (Figure 6). The CaMV 35S promoter activity was not influenced by any of these growth hormones (data not shown).

Identification of *cis*-Acting Regulatory Elements Involved in the Wound Response

Regulatory elements responsible for the wound inducibility of the *nos* promoter were identified by studying effects of various deletions of the 5′-noncoding sequences on the wound response (Figure 8). Deletion of the DNA sequences upstream of −130 did not eliminate the wound response effect; however, the wound-induced level is much lower in the deletion mutant −130 that lacks the upstream region containing a positive modulator (Ebert et al., 1987; Mitra and An, 1989). Further deletion of the 29 bp between −130 and −101 abolished the promoter activ-

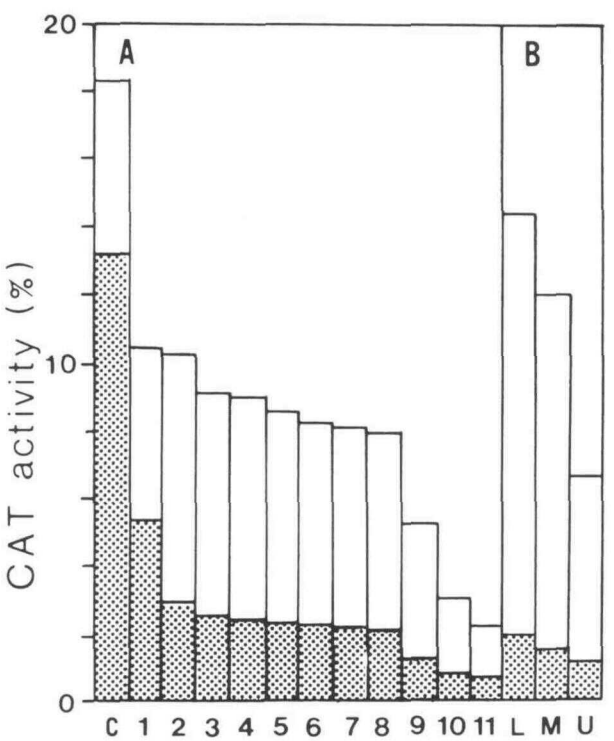

Figure 4. Wound Induction of Various Vegetative Organs.

Samples from an 11-leaf-stage plant grown on MS agar medium were floated on MS medium for 24 hr, and CAT activity was measured from samples before (▩) and after treatment (▩ + □).
(A) c, cotyledon; 1 to 11, leaves numbered from bottom.
(B) L, lower stem; M, middle stem; U, upper stem.
One percent CAT activity is equivalent to 0.021 unit/g of total soluble protein. One unit of CAT enzyme catalyzes acetylation of 1 nm of chloramphenicol per minute at 37°C.

Table 1. CAT Activity in Leaves from Various Locations before (C) or after Wound (W) and 2,4-D (H) Induction

	Top			Middle			Bottom		
Plant Number	C	W	H	C	W	H	C	W	H
	(units/g of total soluble protein) × 1000								
1	0	185	466	40	353	552	62	424	626
2	0	116	632	2	168	737	162	305	1145
3	2	305	928	11	327	1166	46	569	1365
4	0	55	302	8	76	473	15	105	798
5	0	42	84	3	76	97	10	78	512
Average	0	141	482	13	200	605	59	296	889

Greenhouse-grown transgenic tobacco plants carrying the *nos-cat* fusion were assayed by incubating pooled samples of sections from three leaves at each position for 24 hr. Top, unexpanded leaves from upper region of plants; Middle, fully expanded leaves from the mid-region of plants; Bottom, fully expanded leaves from the lower region of plants. One unit of CAT activity converts 1 nmol of chloramphenicol in 1 min at 37°C.

ity in both unwounded and wounded tissues, suggesting that the wound response element is located downstream of −130. To investigate the role of the downstream region that contains the CAAT and TATA box elements on the wound response, a hybrid promoter was constructed by fusing the *nos* mutant −101 to the upstream control region of the chlorophyll *a/b*-binding protein (*cab*) promoter (Ha and An, 1988). Transgenic tobacco plants carrying this hybrid promoter exhibited similar levels of CAT activity before and after wounding the leaves (Figure 9). Thus, the *nos* downstream region alone is not sufficient for the wound response. This conclusion was further supported by the observation that internal deletion of either the CAAT or TATA box region from the *nos* promoter did not alter the wound response (Figure 8). Therefore, at least a part of the control elements responsible for the wound induction must be located between −130 and −101 where the 10-bp *Z* element and a positive regulatory element have been previously proposed (Ebert et al., 1987; Mitra and An, 1989). Internal deletion of the DNA sequence carrying the *b* element (between −112 and −101) alone did not affect the wound response, whereas deletion of the *Z* element (between −126 and −116) abolished the wound-inducible promoter activity (Figure 8). All the deletion mutants (−155, −130, ΔTATA, ΔCAAT, Δ*b*) that exhibited wound response were further induced by auxin during the wound induction (Figure 8). These results suggest that the *Z* element or an element overlapping with this sequence is involved in both wound and auxin induction of the *nos* promoter.

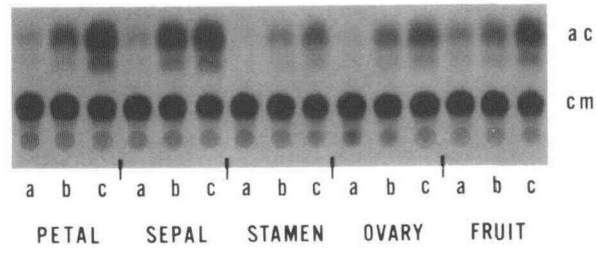

Figure 6. Wound Induction of Reproductive Organs.

CAT activities driven by the *nos* promoter were measured before (a) and after wounding without (b) or with (c) 0.2 μg/mL 2,4-D. The assay conditions were identical to those in Figure 2 except that 20 μg of total soluble protein was used to standardize samples. cm, chloramphenicol; ac, acetylchloramphenicol.

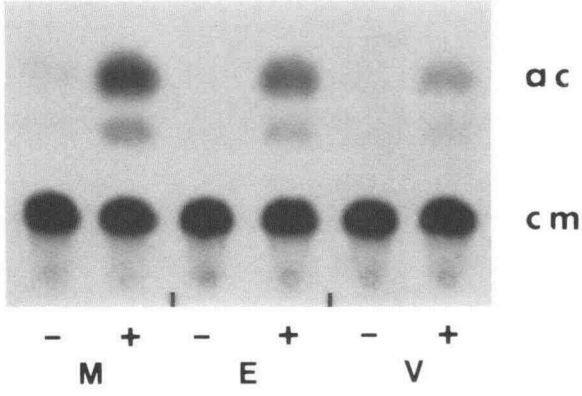

Figure 5. Wound Induction of Leaf Tissues.

CAT activities driven by the *nos* promoter were measured before (−) and after (+) incubation of mesophyll (M), epidermal (E), and midvein (V) tissues isolated from five independently transformed plants. The wound induction and CAT assay conditions were identical to those described in Figure 2. cm, chloramphenicol; ac, acetylchloramphenicol.

Table 2. CAT Activity in Various Reproductive Organs before (C) or after Wound (W) and 2,4-D (H) Induction

	Petal			Sepal			Stamen			Ovary			Fruit		
Plant Number	C	W	H	C	W	H	C	W	H	C	W	H	C	W	H
	(units/g total soluble protein) × 1000														
1	0	8	41	5	73	156	0	6	52	0	10	19	1	14	32
2	1	31	80	1	3	18	0	2	4	0	2	4	1	48	60
3	5	34	152	5	105	170	0	13	38	0	26	50	6	54	84
4	14	27	101	1	30	52	8	64	98	0	36	74	14	42	124
5	13	32	153	10	106	197	0	20	85	0	17	40	14	51	68
Average	7	26	105	4	63	119	2	21	55	0	18	37	7	42	74

Samples were treated as described in the legend to Figure 6. One unit of CAT activity converts 1 nmol of chloramphenicol in 1 min at 37°C.

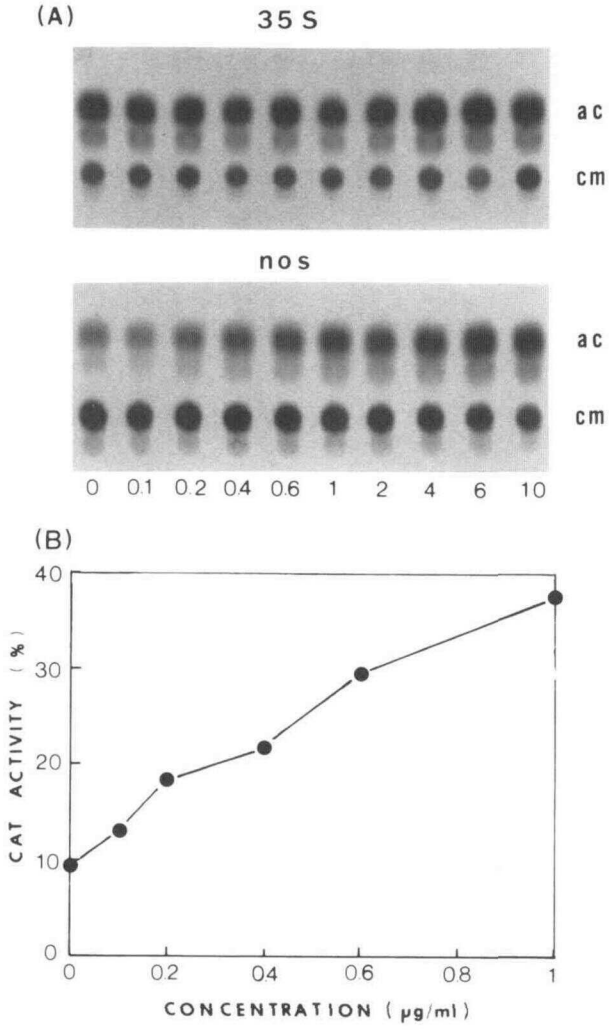

Figure 7. Effect of 2,4-D on Wound Response of the *nos* Promoter.

Leaf sections were incubated for 24 hr in MS medium containing various concentrations of 2,4-D. CAT activity was measured using 4 μg of total soluble protein to standardize samples.

(A) Autoradiograms of thin-layer chromatography plates showing conversion of chloramphenicol (cm) to acetylchloramphenicol (ac) by either CaMV 35S promoter or *nos* promoter activity. Numbers along bottom indicate 2,4-D concentration in micrograms per milliliter.

(B) CAT activities driven by the *nos* promoter are shown as percent conversion of chloramphenicol to acetylchloramphenicol. One percent CAT activity is equivalent to 0.021 unit/g of total soluble protein.

DISCUSSION

Nopaline Synthase Promoter Activity Is Inducible by Wounding

We have shown that both mRNA level and enzyme activity of the *cat* reporter gene were significantly induced in transgenic tobacco leaves that carry the *nos-cat* gene fusion. However, CaMV 35S promoter-driven CAT activity was not altered by the wound treatments. Because both chimeric molecules were constructed with the same reporter and terminator sequences differing only in the promoters, the increase in CAT mRNA and enzyme activity is unlikely due to stability or processing of the gene products but is probably the result of promoter activity. It was previously demonstrated that the *cat* reporter system can be useful to study other wound-inducible promoters (Thornburg et al., 1987; An et al., 1989; Logemann et al., 1989).

It was observed in preceding studies that the basal level of *nos* promoter activity was higher in younger plants and decreased as the plants matured (An et al., 1988). This may account for the difference in the basal level of promoter activity among samples. Although *nos* promoter activity varied depending on the type or location of each organ, all of the vegetative and reproductive organs tested

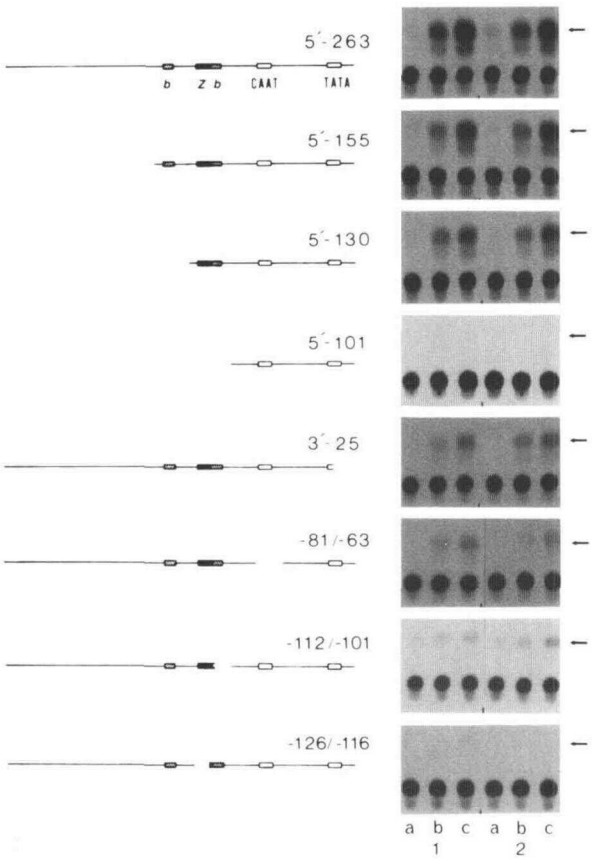

Figure 8. Deletion Analysis of the Wound-Inducible and Auxin-Inducible Element in the *nos* Promoter.

Young leaves of greenhouse-grown transgenic tobacco plants carrying either 5′ deletion mutation or internal deletion mutation were assayed as described in Figure 2. CAT activities were measured before (a) and after incubation of leaf slices in MS medium without (b) or with (c) 0.2 μg/mL 2,4-D. Among several plants tested, results from two representative plants for each deletion mutant are shown. Deletion endpoints and schematic diagrams of the mutant promoters are shown on the left. Numbers 1 and 2 below autoradiogram represent independently transformed plants. Open boxes, the CAAT and TATA boxes; closed boxes, the 10-bp *Z* element; slashed boxes, the 8-bp invert repeats; arrows, acetylchloramphenicol.

in this study exhibited the wound-inducible *nos* promoter activity. This response was found both in young plants grown on sterile agar medium and mature plants grown in the greenhouse. Therefore, it can be concluded that the *trans*-acting factor required for induction of the *nos* promoter is present in a wide variety of plant organs.

The *nos* promoter-driven CAT activity was induced to similar levels by either the leaf slice induction assay in sterile water or mechanical wounding of leaves on intact plants. These results demonstrated that the *nos* promoter responds primarily to mechanical wounding regardless of the assay conditions. However, the wound response of sliced leaves was slightly higher in culture medium compared with water, suggesting that a component in the medium may influence the wound response, although the effect was not as significant as that of wound response.

The molecular mechanisms involved in the wound induction of the *nos* promoter seem to be different from those of potato PI promoters because the *nos* promoter was induced within 1 hr upon wounding, whereas the induction of PI promoter was delayed for several hours (Graham et al., 1986). Furthermore, the wound-inducible *nos* promoter activity was observed in a wide variety of organs, whereas the PI promoter was inducible only in specific tissues (Keil, Sanchez-Serrano, and Willmitzer, 1989).

The Wound Response Is Further Enhanced by Auxin

Addition of auxins into the leaf slices further increased the *nos* promoter-driven CAT activity. The enhancement was concentration dependent. Cytokinins, abscisic acid, and GA_3 did not influence the promoter activity. The auxin effect was found in leaves independent of location and also in various reproductive organs, suggesting that the regulatory mechanism involved in the phytohormone enhancement is present in a wide variety of organs. The fact that T-DNA promoters are active in tumor cells where auxin and cytokinin are abundant may provide an explanation for the physiological basis of our results on the *nos* promoter and the recent observations that another T-DNA promoter is also inducible by auxin (Langridge et al., 1989).

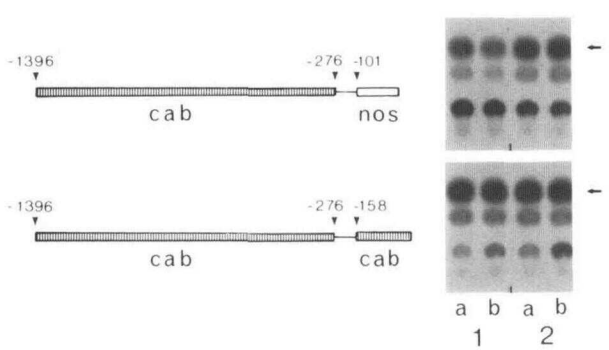

Figure 9. Wound Response of the *cab-nos* Fusion Molecule.

The *cab-nos* molecule contains the *cab* upstream region (between −1396 and −276) and the *nos* downstream region (between −101 and −17). The *cab-cab* molecule is an internal deletion (between −276 and −158) of the *Arabidopsis thaliana cab1* promoter. Construction and detailed structure of these molecules were reported earlier (Ha and An, 1988). Transgenic tobacco plants carrying either *cab-nos* or *cab-cab* molecule were assayed as described in Figure 2. Numbers 1 and 2 below autoradiogram represent independently transformed plants. Arrows indicate acetylchloramphenicol. Sample a, unwounded; sample b, wounded.

Table 3. The Conserved DNA Sequence Elements of Various Auxin-Inducible Promoters

Sequence[a]	Promoter	Position	Reference
GCA--CATACGT	nos	−114	This paper
GCAC-CATGCGT	aux28-B′	−210	Ainley et al., 1988
GCAG-CATGCAC	aux28-B	−285	Ainley et al., 1988
GCAG-CATGCAT	aux22-B	−207	Ainley et al., 1988
GCATTCATACGC	Gmhsp26	−178	Czarnecka et al., 1988
CCA--TATGCCC	SAURs	−130	McClure et al., 1989
GCGT-CACGCGC	mas	−68	DiRita and Gelvin, 1987
GCAN-CATRCRY	consensus		

[a] R, purine; Y, pyrimidine; N, any nucleotide; -, a gap.

Immediate Upstream Region of the *nos* Promoter Is Involved in the Wound Induction and Auxin Induction

Deletion mutation analysis of the *nos* promoter indicated that the immediate upstream region between −130 and −101 is involved in the wound induction and auxin induction. The DNA sequence downstream of −101 that carries the CAAT and TATA box region was unable to respond either by itself or in connection with the *cab* upstream promoter region. It was also observed that deletion of DNA sequences carrying the *Z* element nullified the response, whereas deletion of surrounding sequence elements reduced but did not abolish wound- and auxin-induced expression of the *nos* promoter. Therefore, the *Z* element or an element in this region is essential for induction.

Comparison of DNA sequences between the *nos* promoter and the 5′ control regions of other auxin-inducible genes (Ainley et al., 1988; Czarnecka et al., 1988; McClure et al., 1989) exhibited a conserved sequence homologous to the *nos Z* element (Table 3), suggesting that the consensus sequence may be the key element responding to the wounding and auxin. An alternative hypothesis would be that the wound-responsive sequence involves not the *Z* element specifically but another element that partially overlaps with the *Z* element. One possible candidate is a hexameric nucleotide motif, ACGTCA, that shares four nucleotides in common with the *Z* element. The *hex* element is located in this important regulatory region of several plant promoters and interacts with a nuclear protein factor (Katagiri, Lam, and Chua, 1989; Tabata et al., 1989). Site-specific mutagenesis of the *nos* immediate upstream promoter region is underway to further understand structure and elucidate the function of the wound- and auxin-inducible regulatory element.

METHODS

Bacterial Strains and Plant Materials

Escherichia coli host strain MC1000 (*ara; lec, lac, gal, str*) (Casadaban and Cohen, 1980) was used as the recipient for routine cloning experiments. The *Agrobacterium* strain LBA4404 (Hoekema et al., 1983) containing the Ach5 chromosomal background and a disarmed helper Ti plasmid, pAL4404, was used for the transformation of *Nicotiana tabacum* cv Xanthi (An, Watson, and Chiang, 1986a). At least five independently transformed plants were regenerated for each promoter mutant that was described earlier (An et al., 1986b; Ebert et al., 1987). Plants at the 11-leaf stage, grown on MS agar medium (Murashige and Skoog, 1962), were used for the experiments shown in Figure 4. All other results were obtained from greenhouse-grown plants. Decigram quantities of epidermal, mesophyll, and midvein tissues were isolated by dissection of leaves. More than 99% purity was observed for each tissue type by microscopic examination.

Leaf Slice Induction Assay

Leaves were cut into approximately 0.5-cm^2 sections and the slices were floated on MS liquid medium containing 3% sucrose, 250 μg/mL cefotaxime, 250 μg/mL carbenicillin, and 50 μg/mL kanamycin. The leaf sections were incubated for 24 hr at 28°C unless otherwise mentioned in the text. CAT activity was measured (An, 1987) using crude extracts standardized to 4 μg/mL (for vegetative organs) or 20 μg/mL (for reproductive organs) of total soluble protein. Reaction time was 20 min.

RNA Dot-Blot Hybridization

Total RNA from the greenhouse-grown plants was prepared as described previously (Mitra and An, 1989). Briefly, frozen plant materials were ground in liquid nitrogen, and 2 mL of extraction buffer (8 M guanidium chloride, 20 mM Mes, 20 mM EDTA, and 50 mM β-mercaptoethanol) per gram of sample was added. The mixture was extracted with phenol:chloroform:isoamyl alcohol (24:24:1) twice, and the RNA was precipitated with ethanol and acetic acid. Eight and 4 μg of total RNA were applied to the Zeta-Probe membrane (Bio-Rad, Hercules, CA) and hybridized with ^{32}P-labeled random-primed DNA fragment containing the *cat* gene. Hybridization was at 65°C in a solution containing 100 mM Tris, pH 7.5, 1 M NaCl, 2% SDS, 1% BSA, 1% PVP, 1% Ficoll, and 100 μg/mL denatured salmon sperm DNA. Membranes were washed successively for 15 min each in 2 × SSC, 0.5 × SSC, and 0.1 × SSC containing 0.1% SDS at room temperature before autoradiography.

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