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Organ-Specific and Developmental Regulation of the Nopaline Synthase Promoter in Transgenic Tobacco Plants[1]

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ABSTRACT

Control regions of the nopaline synthase (*nos*) gene have been widely used to express foreign genes in plants since the promoter is active in a wide variety of plant tissues. We report here the characteristics of the *nos* promoter activity in transgenic tobacco (*Nicotiana tabacum*) plants at various developmental stages. The promoter was highly active in the lower parts of a plant and gradually decreased in the upper parts. This vertical gradient was maintained throughout plant growth until the flowering stage when the overall promoter strength decreased significantly in the vegetative organs. However, in various flower organs, the *nos* promoter activities increased dramatically. Higher activity was observed in calyx, corolla, and stamens although the maximum promoter activity in each organ was found at different stages of flower development. The promoter activity in pistils was low and gradually increased in the ovaries after anthesis. In developing fruits, the *nos* promoter activity was strongly induced during the mid-stage of embryogenesis. These results indicate that the expression of the *nos* promoter is developmentally regulated and organ specific in transgenic tobacco plants.

Several *Agrobacterium* genes located within the T-DNA[2] of Ti plasmids are readily expressed in plant tissues upon transfer to the plant chromosome (9, 14). The nopaline synthase (*nos*) gene is located at the right border of the T-DNA and is expressed in several plant species (8, 18). The *nos* protein catalyzes the synthesis of nopaline from arginine and α-ketoglutaric acid (11). Recently the gene and its control regions have been widely used for transformation studies. Observations of *nos* activity were often used for the indication of stable transformation of plants (22). Furthermore, the promoter and terminator are widely used to express foreign genes such as drug resistance markers for the construction of plant selectable markers (4, 6, 12, 15). Three *cis*-acting regulatory elements necessary for efficient expression in tobacco calli have been identified in the 5′ control region of the *nos* promoter (3, 10). The TATA and CCAAT box homologies, that are found in many eukaryotic promoters, are also important for efficiency of the *nos* promoter activity. Deletion of each element resulted in about 10-fold reduction of the promoter strength in cultured tobacco cells. In addition, an upstream regulatory region located about 20 bp upstream from the CCAAT box region was essential for the *nos* promoter to function. Deletion of the upstream regulatory region abolished the promoter activity. The *nos* gene has also been used as a control in several experiments for studying *cis* regulatory elements since expression of the *nos* gene was considered to be constitutive in plants (16, 23–25). However, it has not been adequately studied whether this T-DNA gene is indeed constitutive or regulated by developmental or environmental factors. In this study, we report that the *nos* promoter activity is organ specific and developmentally regulated.

MATERIALS AND METHODS

Bacterial Strains. *Escherichia coli* strain MC1000 (7) was used as the host for routine cloning experiments. *Agrobacterium* strain LBA4404 (17), carrying an avirulent helper Ti plasmid pAL4404, was used for maintenance of plasmids and transformation of tobacco cells.

Plasmid Construction. pGA658 and pGA663 were made by inserting the 796 bp *Bam*HI-*Bgl*II fragment of pGA425 (4) carrying the coding region of chloramphenicol acetyltransferase (*cat*) gene into the *Bgl*II site of pGA642 and pGA643 (2), respectively. pGA642 is an expression Ti-plasmid vector which

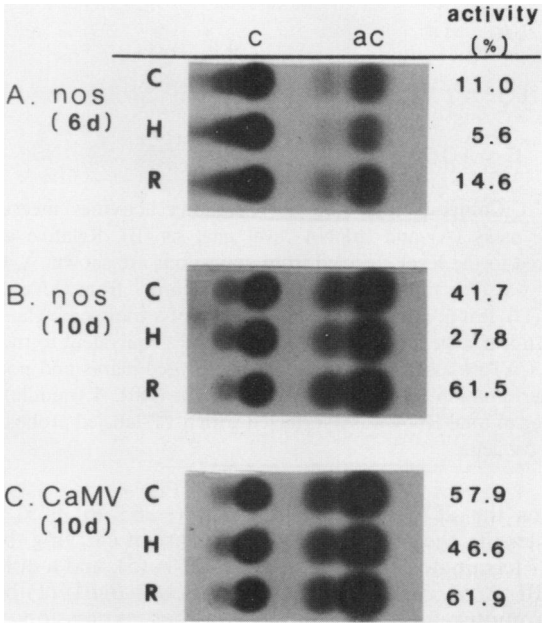

FIG. 1. Differential expression of *nos* promoter in seedlings. Cotyledons (C), hypocotyls (H), and roots (R) were harvested from 50 young seedlings (6-d or 10 d-old) containing *nos-cat* (A and B) or CaMV-*cat* (C). The extracts equivalent to either 4 μg of total protein for the *nos* promoter or 1 μg for the CaMV promoters were used for CATase assay. Enzyme activity is presented as percent conversion of chloramphenicol (c) to acetylchloramphenicol (ac).

[1] Supported in part by a grant from the National Science Foundation (DCB-8616018).

[2] Abbreviations: T-DNA, transfer DNA; Ti, tumor-inducing; CATase, chloramphenicol acetyltransferase; bp, base pair; SSC, standard saline citrate.

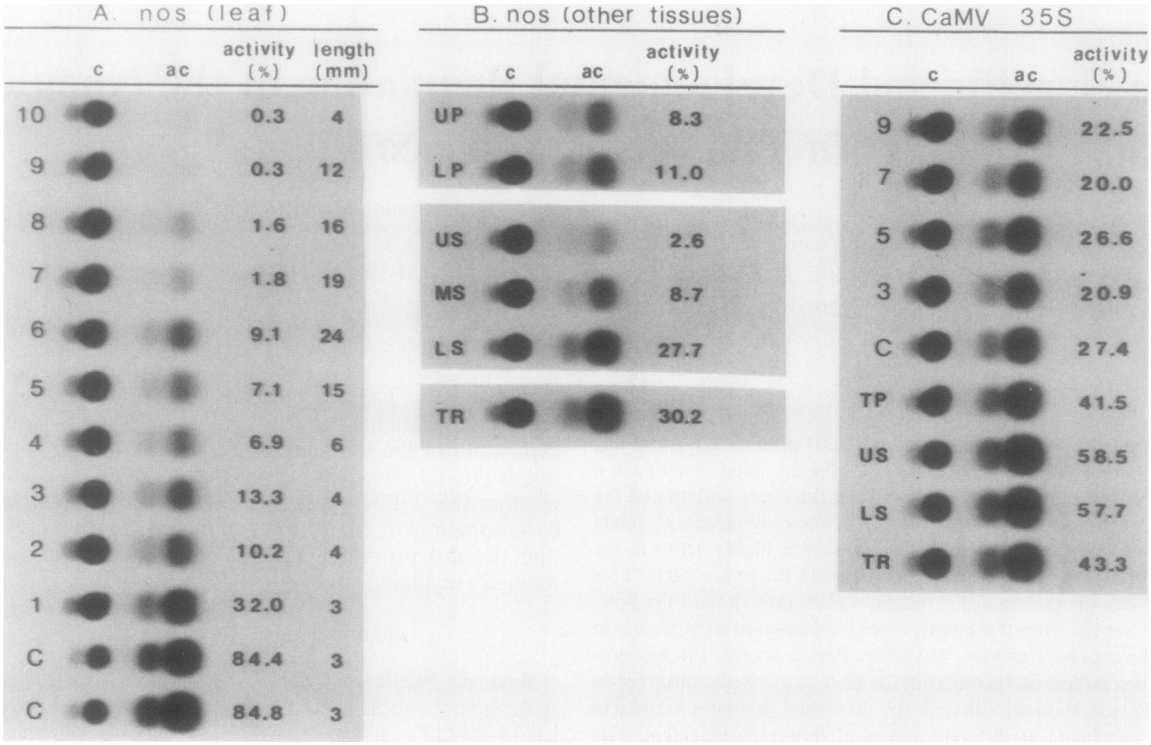

FIG. 2. Promoter-activity gradient in a young tobacco plant. Various organs from a 10-leaf stage plant carrying either *nos-cat* (A and B) or CaMV-*cat* (C) were harvested for CATase assay as described in Figure 1. CATase activity was measured from the individual leaf (numbered from the bottom to the top) and from pools of cotyledons (C), petioles (UP, upper petioles; LP, lower petioles; TP, total petioles), stems (US, upper stems; MS, middle stems; LS, lower stems), and roots (TR). Length of leaves and cotyledons is indicated as mm.

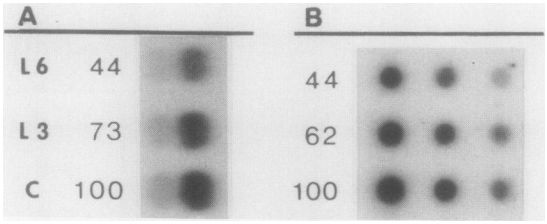

FIG. 3. Comparison of the *nos* promoter activities measured by CATase assay (A) and mRNA level analysis (B). Relative activities compared to the level detected from cotyledons are shown. A, CATase activity was determined as described for Figure 1 from three different organs (L6, leaf 6; L3, leaf 3; C, cotyledons) of a transgenic plant grown to the 16-leaf stage in MS medium; B, samples equivalent to those used for the CATase assay were harvested from three plants and pooled for the determination of *cat* mRNA level. Eight (left), 4 (middle), and 2 (right) μg of total RNA was hybridized with a ^{32}P-labeled probe containing the *cat* gene.

contains the 288 bp DNA fragment (−263 to +25) of *nos* promoter (8), the 740 bp *Cla*I-*Nde*I fragment carrying the transcript 7 terminator of the octopine T-DNA (5), and a polylinker (*Hin*dIII, *Xba*I, *Sst*I, *Hpa*I, *Kpn*I, *Cla*I, and *Bgl*II) (1) between the promoter and terminator. Another expression vector, pGA643, contains a 419 bp DNA fragment (−418 to +1) of the 35S promoter of CaMV strain 1841 (13) in place of the *nos* promoter of pGA642.

Chloramphenicol Acetyltransferase (CATase) Assay. CATase activity was measured as described previously (2). Plant extract equivalent to 4 μg of total protein was used for *nos* promoter activity measurements; 1 μg of total protein was used for the CaMV 35S promoter. The reaction was carried out at 37°C for 20 min. The rates of reaction were in a linear range over the time of the incubation between 0 to 40% of substrate conversion.

Dot Blot Hybridization. Total RNA from various plant parts was prepared as described (20). 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 g sample was added. Eight, 4, and 2 μg of total RNA were applied to a Gene Screen Plus membrane (New England Nuclear) 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 twice with 2× SSC (1× SSC is 0.15 M NaCl and 0.015 M Na-citrate) containing 1% SDS at 65°C for 45 min, and then twice with 0.1× SSC at 65°C for 30 min prior to autoradiography. Dot-blotting was carried out by using a Bio-dot apparatus (Bio-Rad) under conditions recommended by the manufacturer.

RESULTS

Two binary plasmids were constructed for this study. The first molecule, pGA658, contains a transcriptional fusion between the *nos* promoter, *cat* coding region, and the transcript 7 terminator. The second molecule, pGA663 contains a similar fusion except that the *nos* promoter was replaced with the CaMV 35S promoter. These molecules were stably transferred into tobacco plants via the *Agrobacterium* co-cultivation method. Kanamycin resistant transformants were selected and those primary regenerants containing CATase activity were further grown in the greenhouse. Seeds were harvested from the self-pollinated plants and seedlings from surface-sterilized seeds were grown on an MS agar

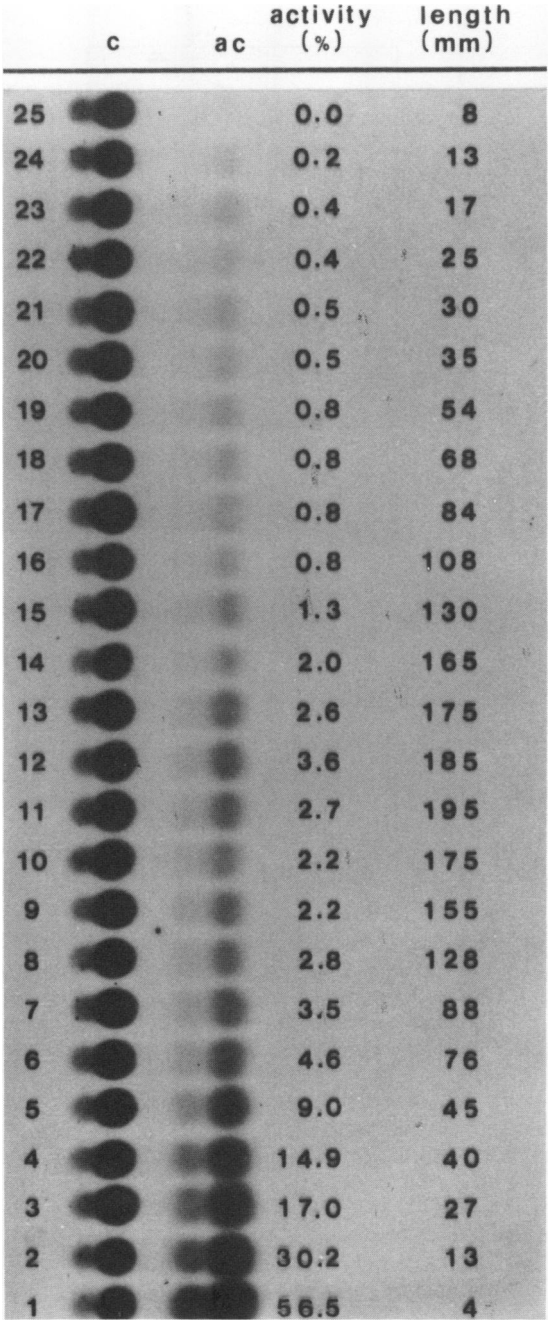

FIG. 4. Promoter-activity gradient in a greenhouse-grown plant. The leaves were numbered from the bottom to the top. The data are presented as described for Figure 2.

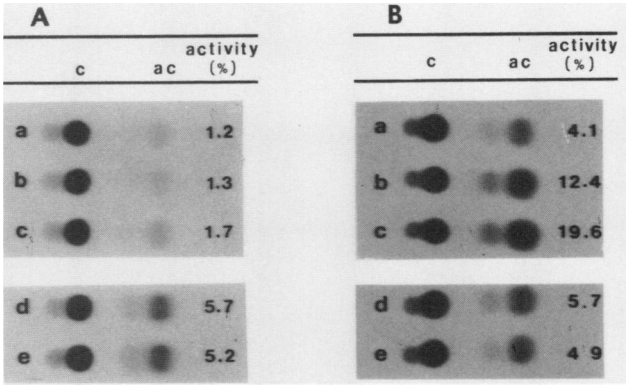

FIG. 5. Organ-specific expression in leaves at different positions. The middle (A) and lower (B) leaves were harvested from the greenhouse-grown plants (Fig. 4). CATase activities were measured from basal (a), middle (b), and apical (c) portion of leaf blades, and from bottom (d) and middle (e) of midribs.

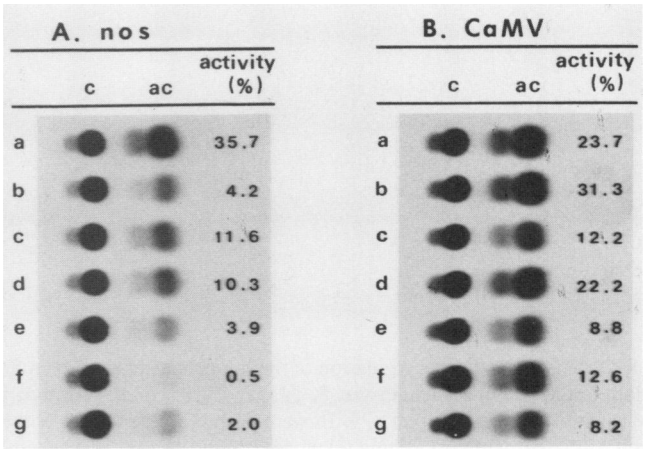

FIG. 6. Organ specific expression in flower. Transgenic tobacco flowers carrying either *nos* (A) or CaMV 35S (B) promoter were sampled at the stage immediately before anthesis. CATase activities were measured from the corolla (a), calyx (b), anthers (c), filaments (d), stigma (e), styles (f), and ovary (g).

medium (21) containing 50 μg of kanamycin per mL and 3% sucrose under light (12 h/d, 50 $\mu E/m^2 \cdot s$). Several independent transgenic tobacco plants for each construct were used for this study.

Differential *nos* Promoter Activity During Seedling Development. The *nos* promoter activity was monitored in 6-d-old and 10-d-old light-grown seedlings by measuring the CATase activity using equivalent amounts of protein extracted from roots, hypocotyls, and cotyledons (Fig. 1, A and B). The promoter activity was about two- to threefold higher in roots compared to hypocotyls. An intermediate level of the promoter activity was observed in cotyledons. This pattern of expression has been observed consistently in all the plants that were examined. The 10-d-old seedlings contained overall fourfold higher promoter activity compared to the 6-d-old seedlings, but the relative promoter strength between different organs remained the same. Transgenic seedlings carrying the 35S promoter-*cat* fusion did not exhibit a significant differential expression in these organs (Fig. 1C).

Developmental Regulation of the *nos* Promoter. In order to examine whether the *nos* promoter is also differentially expressed during further growth, transgenic tobacco plants containing the *nos-cat* fusion molecule were grown aseptically in an MS medium supplemented with 3% sucrose and CATase activity was measured in various tissues (Fig. 2A). The *nos* promoter was highly active in cotyledons and lower leaves whereas it was weakly expressed in upper leaves. As shown in Figure 2B the promoter was also active in other tissues and the promoter-activity gradient was maintained in petioles and stems. Plants at different growth stages displayed a similar trend that the *nos* promoter is more active in older tissues. There was no correlation between the size of the leaf and the promoter strength. The largest leaf of the 10-leaf stage plant was the sixth, which carried about 10% of CATase activity compared to the cotyledons and 30% of the lowest leaf. Such a profound difference in the promoter activity was not found in transgenic tobacco plants carrying the CaMV 35S-*cat* molecule (Fig. 2C). The CaMV 35S promoter was almost equally

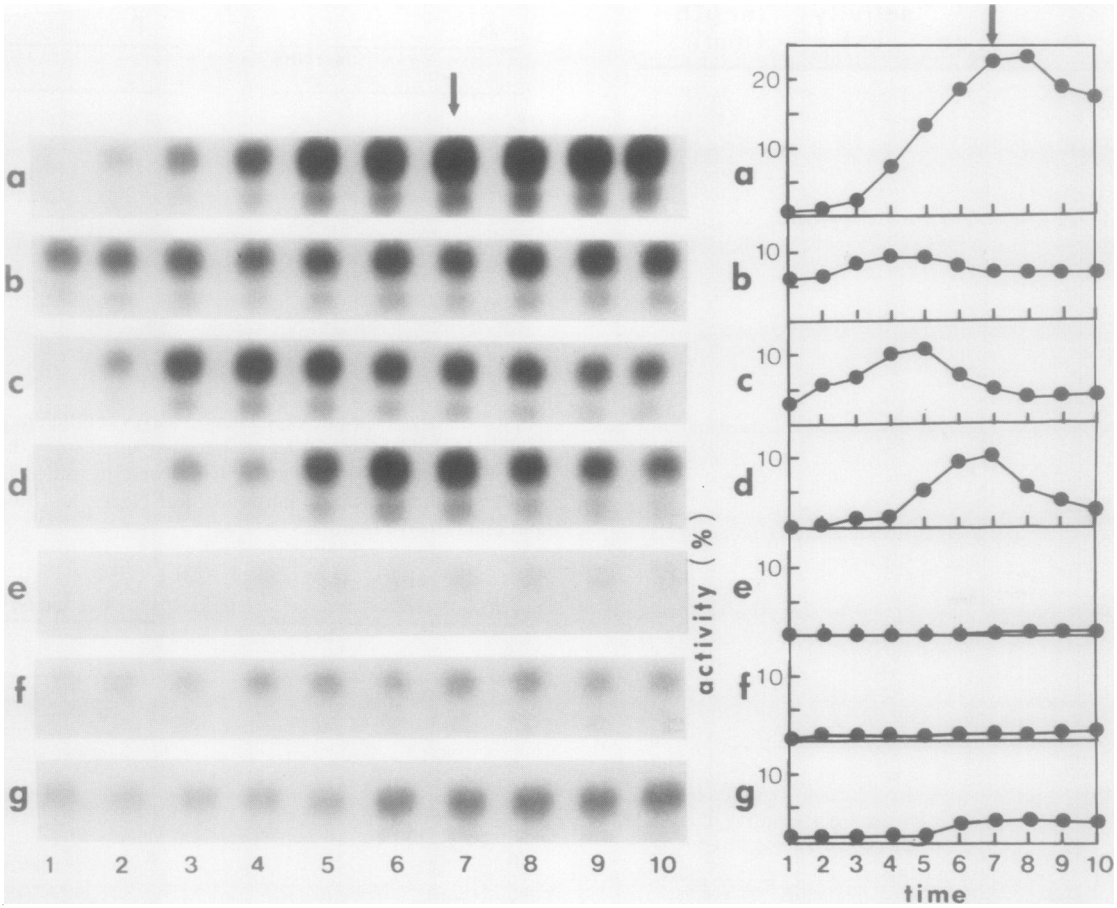

FIG. 7. Developmental regulation of the *nos* promoter activity in flower. Flowers were sampled at various stages. Samples 1 to 6 were the flowers before anthesis. Flower length was 8, 14, 17, 27, 35, and 45 mm at the stages from 1 to 6. Sample 7 was a flower at the day of anthesis and is indicated by arrows. Samples 8, 9, 10 were flowers 1, 2, 3 d after anthesis, respectively. CATase activities were measured from corolla (a), calyx (b), anthers (c), filaments (d), stigma (e), styles (f), and ovary (g). The left hand side of the figure shows an autoradiogram of the CATase assay from a single experiment. The right hand side of the figure represents change of the CATase activity obtained by averaging three independent experiments.

active in the different leaves regardless of the position and about two- to threefold stronger in petioles, stems, and roots than in leaves.

Transcriptional Regulation of the *nos* Promoter. In order to verify that the CATase activity is reflective of the mRNA levels, total RNA was prepared from various tissues and hybridized with a ^{32}P-labeled DNA probe containing the *cat* coding region. The result shown in Figure 3 indicates that the relative amount of *cat* mRNA in leaves and cotyledons is in agreement with the CATase activity found in the corresponding organ.

Differential *nos* Promoter Activity in Greenhouse Grown Plants. The above results were obtained from sterile plants grown under a relatively low light intensity on a synthetic growth medium supplemented with a high concentration of sucrose. To test whether a similar differential expression is maintained in plants grown under more natural conditions, greenhouse grown plants were examined. As observed with young plants grown in the laboratory, the greenhouse grown plants displayed an overall similar distribution of the *nos* promoter activity (Fig. 4). At least several hundredfold higher promoter activity was detected in the lowest leaf compared to the upper leaves. Such a gradient was also found in petioles and stems (data not shown).

In order to examine whether the *nos* promoter is differentially expressed depending upon the position or tissue types within a leaf, two leaves from the greenhouse-grown plant shown in Figure 4 were subsampled and CATase activity was measured. The results indicated that the promoter activity was differentially distributed within a leaf. The midribs had four- to fivefold stronger expression than the leaf blades (Fig. 5A). In the lower leaves, however, the promoter was more active in leaf blades than in midribs (Fig. 5B). The results also showed that the apical parts of leaf blades contained higher CATase activity compared to the basal parts. It appears that the higher promoter activity found in the lower leaves is mainly associated with cells in leaf blades.

Organ Specific Expression in Flowers. The typical promoter-activity gradient was retained in tobacco plants throughout the entire growth stage. However, the overall strength of the promoter was significantly decreased at the flowering stage. There was very little CATase activity in the upper part of the plant including the flower buds. However, the promoter activity in flowers significantly increased during flower development and became highest at anthesis (Figs. 6A and 7). In flowers most of the *nos* promoter activity was associated with calyx, corolla, and stamens. At anthesis, the corolla retained several hundredfold higher CATase activity than styles which showed the lowest promoter activity, whereas the difference of the CaMV 35S promoter activity in flower organs was within fourfold range (Fig. 6B). The accumulation pattern of the CATase activity driven by the *nos* promoter was significantly different in various flower organs (Fig. 7). In calyx, the *nos* promoter activity was slightly increased at the early flowering stage and remained at an

almost identical level after anthesis (Fig. 7b). In corolla and stamens, the promoter activity was strongly regulated during development. The *nos* promoter activity in corolla increased rapidly during flower maturation and remained at the higher level after anesthesis. The promoter activity decreased in corolla during flower senescence (Fig. 7a). Similarly, the promoter was most active in filaments at anthesis and gradually decreased afterward (Fig. 7d). In anthers, the highest promoter activity was observed a few days before anthesis. After anthesis, pollen was released and the promoter activity decreased (Fig. 7c). In pistils and pedicels a low level of the promoter activity was observed before anthesis and slightly increased afterward (Fig. 7, e, f, and g).

Organ Specific Expression in Fruit. As observed in flowers, the *nos* promoter displayed organ-specific and developmentally regulated expression in tobacco fruits during seed development (Fig. 8). In seed and placenta, the promoter activity was low at the early stage of embryogenesis (until 9 d after pollination). However, the promoter became at least 10-fold more active during the midstage of embryo development and the activity gradually decreased at the late stage. A similar increase in the promoter activity was also observed in pericarp, although the level was much lower compared to the other fruit organs. The promoter activity in calyx was high during the early stage and decreased gradually at midembryogenesis stage.

DISCUSSION

The T-DNA genes of *Agrobacterium* are relatively inactive in bacteria but they become active in plant cells upon the transfer, indicating that plant regulatory factors which control gene expression are able to recognize these foreign genes located in the T-DNA. Characteristics of T-DNA gene expression may represent those of a certain class of plant genes. Studying the regulatory mechanism controlling a T-DNA gene expression as a model system of plant regulatory elements has several advantages since the structures of the T-DNA genes are well characterized and the flanking regions are short.

T-DNA genes are expressed in the crown-gall tumors in which a high level of plant growth substances are produced by the T-DNA genes. However, the characteristics of the individual T-DNA genes in differentiated plant cells are not well understood. Although the *nos* gene and its control regions are frequently used for plant transformation experiments, the expression pattern of the gene in various organs and developmental stages has not been studied in detail. It has been generally considered that the *nos* gene is expressed constitutively in plants. In this study we have demonstrated that the activity of the *nos* promoter is organ specific and developmentally controlled.

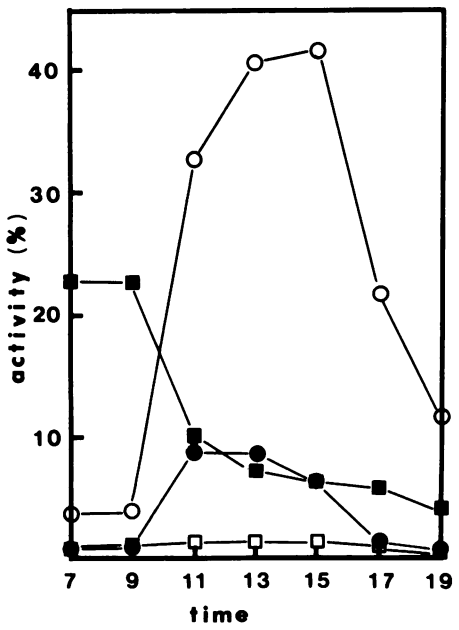

FIG. 8. Developmental regulation of the *nos* promoter activity in fruit. CATase activities were measured from placenta (○), seeds (●), calyx (■), and pericarps (□) from fruits developed for 7 to 19 d after pollination.

The promoter activity varied in different tissues of young seedlings. As the seedlings matured, the overall promoter strength increased while maintaining the pattern of the differential activity. When plants developed to multileaf stages, the promoter strength was maintained at a high level in the lower parts, but it gradually decreased toward the upper parts of plants. This vertical gradient was found in leaves, stems, and petioles, and was maintained throughout the growth until the flowering stage when the promoter activity decreased in all the vegetative organs.

The promoter activity was differential within a leaf and the expression pattern of each leaf was different depending on position. Midribs were primarily responsible for the activity in middle leaves whereas leaf blades retained a high activity in the lower leaves. Therefore, the vertical promoter gradient found in tobacco leaves is mainly due to the promoter activity in leaf blades especially in the apical parts of the leaf.

Young flowers retained a weak *nos* promoter activity. Most of the activity was found in the calyx. However, expression of the promoter increased rapidly in stamens and corolla during flower development and decreased upon senescence. Such a temporal gene expression was also found during fruit development. The promoter activity remained at a low level in placenta and seeds until the midstage of embryogenesis when the activity dramatically increased, whereas the promoter was active in calyx during the early stage and became weak at midembryogenesis.

The developmental regulation of the *nos* promoter observed in this work is probably not due to positional effects of the T-DNA insert in plant chromosomes since the same results were obtained from all the transgenic plants. The *cat* transcripts generated by either *nos* or CaMV promoter are almost identical and the level of expression driven by the CaMV 35S promoter did not show such developmental expression. Therefore, it is unlikely that the temporal and special expression of the *nos* promoter reported here is due to the differential stability of *cat* transcript or protein.

It is not known what factors control the complex pattern of the *nos* promoter expression in tobacco plants. Generally, the promoter activity was higher in mature organs compared to young organs, suggesting that some growth substances that are involved in maturation may play an important role in regulating the *nos* promoter. Preliminary results indicate that the *cis*-acting sequence controlling the flower specific expression is different from that regulating the vertical gradient in plants (S Ha, G An, unpublished observations), suggesting that the promoter diversity is caused by multiple factors.

Further studies of the mechanisms controlling the *nos* promoter activity during plant development may provide important information about plant gene regulation as studies of regulatory mechanisms of viral gene expression in bacteria and other eukaryotes have provided much information about their host gene regulation. Another T-DNA gene, the transcript 5 gene, has been shown to be tissue-specific (19). This observation and our result suggest that other T-DNA genes may also be developmentally and/or environmentally regulated.

The *nos* promoter has been used for the construction of chimeric genes as dominant selectable markers for transformation of higher plants. Ti-plasmid vectors containing these mark-

ers are currently employed to transform a wide variety of plant species. The observation that *nos* promoter activity is differentially expressed in various organs indicates potential problems in regenerating transformants. Different plant species may exhibit a different pattern of *nos* promoter activity during development. Therefore, the method used for transformation of tobacco using a chimeric selectable marker that is under the control of the *nos* promoter may not be suitable for other plant species.

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