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Differential Expression of the Potato Proteinase Inhibitor II Promoter in Transgenic Tobacco

Su Jin Kim[1], Mi-Yeon Lee[1], Gynheung An[2], and Seong-Ryong Kim[1]*
[1]Department of Life Science, Sogang University, Seoul 121-742, Korea
[2]Department of Life Science and Division of Molecular Life Science, Pohang University of Science and Technology, Pohang 790-784, Korea

We studied temporal and spatial expression patterns of the potato proteinase inhibitor II (PI-II) promoter, using transgenic tobacco (*Nicotiana tabacum* L. cv. Xanthi) plants that carried a fusion between the PI-II promoter and the chloramphenicol acetyltransferase (*cat*) gene. PI-II promoter activity was low when plants were young, but increased as plants grew. In 8-week-old plants, old leaves showed higher activity than young leaves. At flowering stage (ca. 15 weeks), the overall promoter activity was reduced to a lower level except in the petals. Compared with stems or petioles at the flowering stage, the roots and floral organs showed minimal activity for the PI-II promoter. We used several environmental stimuli to examine the induction of the PI-II promoter in different organs. Promoter induction was effected by wounding or methyl jasmonate in stems, petioles, sepals, and leaves. The induction was highest in leaves, as was sucrose-enhanced wound induction. These results suggest that the PI-II gene is temporally and spatially regulated. We also established a transient assay system in tobacco BY2 suspension cells to elucidate the upstream regulatory region of the PI-II promoter. A field strength of 0.75 kV/cm and 400 μF capacitance were optimal electroporation conditions for our transient assay.

Keywords: chloramphenicol acetyltransferase, electroporation, promoter, proteinase inhibitor, transgenic tobacco

In response to various environmental stresses, plants express a group of specific genes that are not usually active under normal growth conditions. Insect attacks induce the systemic accumulation of proteinase inhibitors (PIs), which are insecticidal proteins (Ryan, 1973). In potato leaves, wounding induces two types of PIs: PI-I, harboring chymotrypsin inhibitor activity and PI-II, carrying inhibitor activities for both trypsin and chymotrypsin (Pearce et al., 1982). The PI-II proteins accumulate at high levels in the stolon and tuber, and in leaves damaged through insect attack or mechanical wounding. In leaves, the accumulation of the inhibitor is not restricted to the wounded site, but is also observed in nearby unwounded tissues. This indicates a systemic induction of PI-II proteins (Keil et al., 1989). Stimuli such as pectic polysaccharides, chitosan, systemin, and electrical signals have been suggested as wound factors for the systemic induction of PIs (Bishop et al., 1984; Pena-Cortes et al., 1988; Wildon et al., 1992; Schaller and Ryan, 1995).

The 5' control region of the PI-II gene contains the necessary information about wound responses, while the 3' control region provides strong expression of the gene, probably either by increasing stability or through efficient processing of the mRNA (An et al., 1989; Keil et al., 1989). A lipid-derived molecule, methyl jasmonate (MJ), can act as a volatile signal that induces the accumulation of PIs (Farmer and Ryan, 1990). Methyl jasmonate is a naturally occurring compound in plants from many different families (Anderson, 1989). This compound is of considerable interest because of its biological activities in regulating PIs and vegetative storage proteins (Farmer and Ryan, 1990; Franceschi and Grimes, 1991). The addition of sucrose to wounded leaves further enhances expression of the potato PI-II gene (Johnson and Ryan, 1990). However, it is unknown whether these various factors induce the PI-II promoter by independent regulatory sequences or if some of the regulatory elements are shared by different environmental factors.

Promoter analysis has been conducted for PI-II regulatory sequences. The internal fragment between −625 and −520 was sufficient to confer the response to MJ, wounding, or sucrose when it was placed upstream of the *nos* (nopaline synthase) minimal promoter −101 containing the CAAT-TATA region. The wound response element is located between −625 and −570; the G box sequence (CACGTGG) located

*Corresponding author; fax +82-2-704-3601
e-mail sungkim@ccs.sogang.ac.kr

at −570 is essential for methyl jasmonate response (Kim et al., 1991). The description of the G box sequence (CACGTGG) for MJ response has recently been extended by the identification of a palindromic TGACG element within the LOX1 (lipoxygenase) promoter. This element was essential for jasmonate inducibility, and has been identified as a binding site for bZIP *trans*-acting factors (Rouster et al., 1997). At least two independent sucrose response elements in the region between −570 and −500 also have been identified (Kim et al., 1992). Location of the sucrose-responsive and wound-responsive sequences at separate distinctive regions suggests that the induction mechanism for PI-II by sucrose and wounding might be independent.

In this study, we investigated whether the PI-II promoter displays temporal and spatial expression patterns during growth and development. In addition, we established a transient assay system using the tobacco suspension cell line BY2 in order to elucidate the *cis*-elements controlling the PI-II promoter.

MATERIALS AND METHODS

Bacterial Strains and Plant Materials

For the hosts in routine cloning experiments, we used *Escherichia coli* strains XL1-Blue MRF'{*Δ(mcrA)183, Δ(mcrCB-hsdSMR-mrr)173, end A1, supE44, thi-1, recA1, gyrA96, relA1, lac* [*F' proAB, lacI*^{q}*ZΔM15, Tn10(tet*^{r}*)*]} and MC1000 [F-, *araD139, Δ(araABC-leu)7679, galU, galK, ΔlacX74, thi, rps L(str*^{r}*)*] (Casadaban and Cohen, 1980). Surface-sterilized seeds of transgenic tobacco plants (*Nicotiana tabacum* L. cv. Xanthi) were selected with 200 mg L^{-1} kanamycin, and grown aseptically on an MS agar medium (Murashige and Skoog, 1962) supplemented with 3% sucrose. Five days after germination, the seedlings were moved to a Magenta box containing an MS agar medium without kanamycin. After three weeks, the plants were transferred to pots and grown under greenhouse conditions until they reached the flowering stage at ca.14 to 16 weeks. Samples were harvested at different stages of development. Bright Yellow 2 (BY2) tobacco suspension cells were diluted to 1/30 every 7 d, with an MS medium containing 0.2 mg L^{-1} 2,4-D, and were cultivated at 28°C in the dark on an orbital shaker at 120 rpm (An, 1985; Nagata et al., 1992).

Plant Treatments

Plant tissues were sliced into about 0.5- to 1-cm^2 segments, and wounded with forceps while floating on an MS medium. The tissue segments then were incubated for 20 h at room temperature, either in a sucrose-free MS medium for the wounding evaluation or in a 3% sucrose MS medium for the sucrose-treatment experiment. The pH of the MS medium was adjusted to 5.8. Effects of jasmonic acid were studied by adding MJ to the sucrose-free MS liquid medium, at a final concentration of 0.1 mM. The effect of auxin was tested by using 0.2 mg L^{-1} 2,4-D. The samples were homogenized in a plant extraction buffer (0.5 M sucrose, 0.1% ascorbic acid, 0.1% cystein, 0.1 M Tris) and centrifuged at 15,000 rpm for 5 min. The supernatant then was stored at −20°C. The amount of protein extract was quantified based on the Bradford method, using BSA (bovine serum albumin) as a standard (Bradford, 1976).

Protoplast Isolation and Electroporation

Protoplasts were prepared from three-day-old BY2 cells using 1% cellulase and 0.5% pectolyase (Ebert et al., 1987). A 0.5-mL aliquot of 10^6 protoplasts was electroporated in a buffer (10 mM HEPES, 150 mM NaCl, 6 mM $CaCl_2$, 0.4 M mannitol; pH 5.8) with test DNA and 50 μg of carrier DNA. The solution was preincubated on ice for 10 min. Electroporation was performed with a 200-1,000 μF capacitor (Gene Pulser II, Bio-Rad) at 160 V or 300 V across a 0.4-cm path (Fromm et al., 1985; Kim and Choi, 1998). After incubation in 7 mL of a protoplast culture medium (MS medium containing 0.4 M mannitol and 0.2 mg L^{-1} 2,4-D) for 48 h at 28°C, cells were harvested by centrifugation and used for the CAT assay (An et al., 1988a).

CAT Assay

This assay was conducted with 4 to 100 μg of total protein, and using 100 μL of 0.25 M Tris (pH 7.8), 10 μL 4 mM acetyl-CoA, and 40 nCi ^{14}C labeled chloramphenicol at 37°C for 30 to 60 min (An et al., 1988a). The reaction products were extracted with ethyl acetate, separated by thin-layer chromatography, and autoradiographed. CAT activity was quantified by scanning the autoradiogram with a phosphorimager (BAS1500, Fuji). Six to fourteen experiments with four independently transformed plants were conducted. The promoter strength was measured as relative CAT activity, i.e., as a percentage of the

conversion of chloramphenicol to acetylchloramphenicol.

Construction of Mutant Promoters

Standard procedures were used for recombinant DNA work (Sambrook et al., 1989). The plasmid pGA725, which contains the wild-type PI-II promoter, was used for a PCR template (An et al., 1989). The primer sequences for amplifying between −611 and −500 of PI-II were 5'-GGCTCGAGTATCCTCTTT-3' and 5'-GGGGATCCCTC CTATTCA-3'. The PCR-amplified fragments were ligated into the XhoI-BglII site of pGA785, which contains the truncated *nos* promoter −101, the *cat* reporter gene, and an *Agrobacterium 6b* terminator (Palm et al., 1990). To obtain multimers of the fragment, the plasmid was re-opened with XhoI, and an additional copy of the XhoI-BamHI fragment, which was filled-in by Klenow enzyme, was blunt-end ligated. The sequences of the inserted fragments were confirmed by the dideoxynucleotide chain termination method (Sanger et al., 1977) using a thermosequenase cycle sequencing kit (Amersham).

RESULTS

Activity of the PI-II Promoter at Various Developmental Stages before Flowering

Expression of the PI-II promoter in leaves was inducible by wounding, sucrose, auxin, and MJ (Kim et al., 1992). In our study, temporal and spatial expression patterns of the PI-II promoter were examined, using transgenic tobacco plants that carried a chimaeric fusion between the PI-II promoter and the *cat*-coding region.

PI-II promoter activity was weak when plants were young (Fig. 1, A and B), but it increased as the plants grew (Fig. 1C). Although activity at the 8-week stage was generally higher, it varied significantly among different organs. In eight-week-old plants, promoter activity in old leaves was four to five times higher than in young leaves (Fig. 1C). Promoter activity also was high in stems and petioles, but low in roots.

Interestingly, the overall activity was reduced at the flowering stage (Fig. 1D). The cauliflower mosaic virus (CaMV) 35S promoter was used as a control. This promoter has been known to be highly active in most

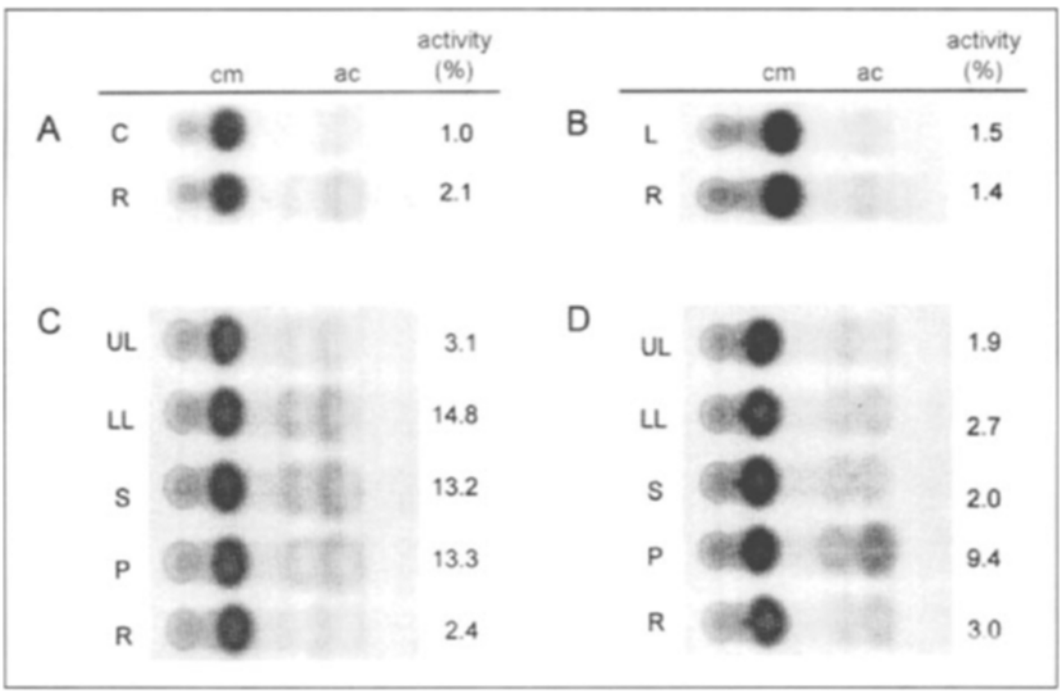

Figure 1. Expression of the PI-II promoter in transgenic tobacco plants at different developmental stages. **A.** 5-day-old stage. **B.** 4-week-old stage. **C.** 8-week-old stage. **D.** flowering stage (ca. 15 weeks). CAT activity was measured from cotyledons (C), leaves (L), upper leaves (UL), lower leaves (LL), stems (S), petioles (P), and roots (R). The results shown are from a representative sample of 6 to 14 experiments with at least four independently transformed plants. Assay conditions: 20 μg of total protein for 30 min incubation period. Enzyme activity is presented as percent conversion of chloramphenicol (cm) to acetylchloramphenicol (ac).

plant organs throughout plant development (Nagy et al., 1985). Comparison of the four- and eight-week-old plants showed no significant differences in CaMV 35S promoter activities (data not shown). This suggests that the temporal and spatial expression of the PI-II promoter reported here is not due to differential stability of the *cat* transcript or protein.

Wound Induction of the PI-II Promoter in Different Organs

Wounding significantly induced activity by the PI-II promoter in all the organs examined (Fig. 2). The level of wound induction was most significant in leaves. When plants were young, the induction was about three-fold. As plants grew, wound induction increased dramatically, reaching a maximum induction of approximately 30-fold in young leaves. In flowering plants, wound induction was reduced in both young and old leaves. In stems and petioles, the promoter activity was also wound-inducible, although induction was not as high as in leaves. Wound induction in stems and petioles also was reduced in flowering plants. Roots had the least significant wound induction. As reported, the CaMV 35S promoter is constitutive in plants (Benfey and Chua, 1990). In our transgenic tobacco plants carrying the CaMV 35S-*cat* molecule, promoter induction by wounding was not

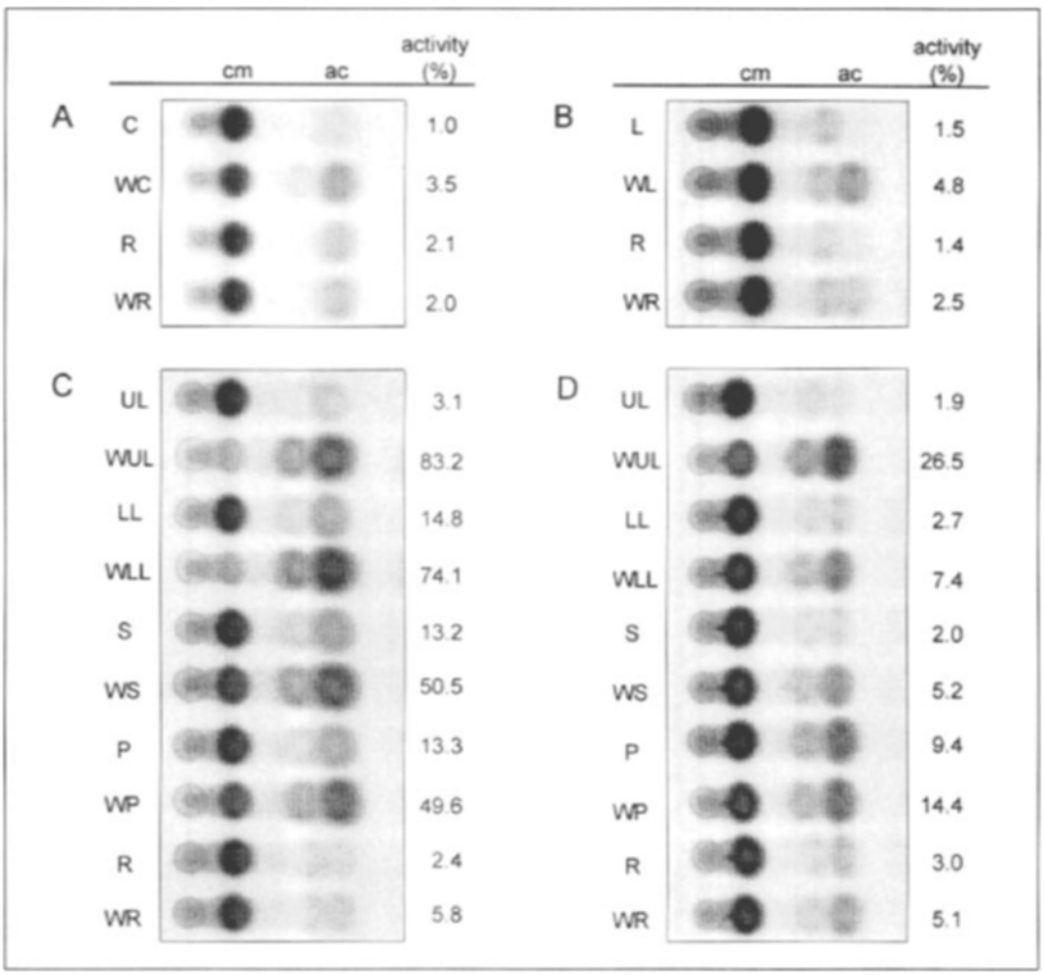

Figure 2. Wound induction of the PI-II promoter in transgenic tobacco plants at different developmental stages. **A.** 5-day-old stage. **B.** 4-week-old stage. **C.** 8-week-old stage. **D.** flowering stage (ca. 15 weeks). Cotyledons (C), leaves (L), upper leaves (UL), lower leaves (LL), stems (S), petioles (P), and roots (R) were sampled after wounding (W) the tissue sections on sucrose-free MS medium for 20 h. The results shown are from a representative sample of 6 to 14 experiments with at least four independently transformed plants. Assay conditions: 20 μg of total protein for 30 min incubation period. Enzyme activity is presented as percent conversion of chloramphenicol (cm) to acetylchloramphenicol (ac).

significant (data not shown).

Effects of Wounding, Sucrose, and MJ on PI-II Promoter Activity at the Flowering Stage

To further test the effects of our wounding, sucrose, and MJ treatments, we examined PI-II activity at the flowering stage. Without any treatment, overall activity was low in both vegetative (Fig. 3) and reproductive organs (Fig. 4). The high level of induction by wounding was displayed in young leaves in the upper portions; the level of induction was less in the lower leaves, stems, petioles, and roots. Induction of the PI-II promoter by wounding was most significant in the sepals and petals (Fig. 4). Sepals showed more than 10-fold enhancement by wounding (Fig. 4).

Both sucrose and MJ also enhanced promoter expression. In leaves, the wound-induction was significantly elevated by the addition of sucrose (Fig. 3). Petioles and sepals also displayed sucrose-enhancement, but other organs were not affected by sucrose. The MJ effect in leaves was not as significant as that of sucrose. Interestingly, the effect of MJ was more significant than for sucrose in stems, petioles, and petals. Sepals showed the most significant induction by wounding, sucrose, and MJ among all plant parts. Here, about a 12-fold induction by wounding and a 20-fold induction by either sucrose and MJ were

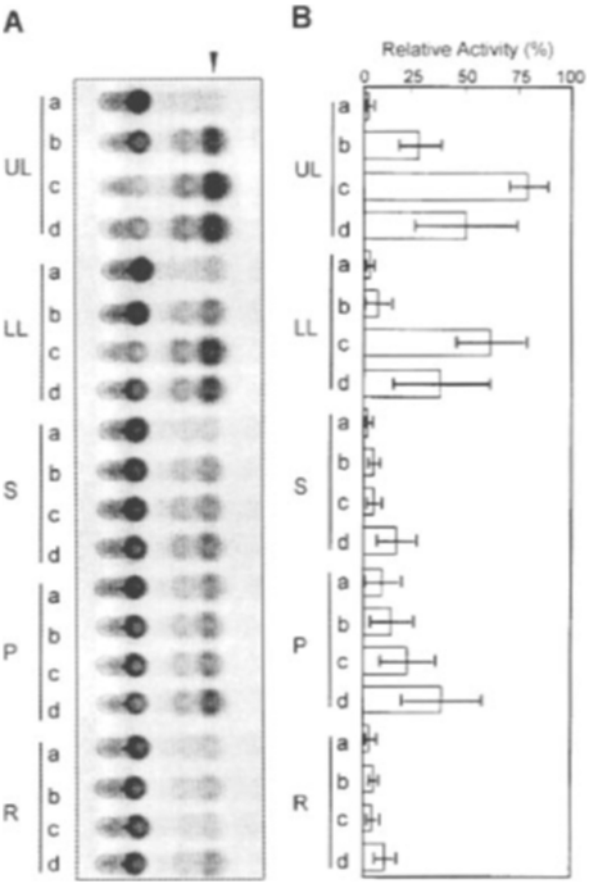

Figure 3. Induction of the PI-II promoter at the flowering stage by wounding, sucrose, and MJ. Upper leaves (UL), lower leaves (LL), stems (S), petioles (P), and roots (R) were sampled before (a) or after wounding the tissue sections on MS medium (b), MS medium with 3% sucrose (c), or MS medium with 0.1 mM MJ (d) for 20 h. **A.** Analysis of CAT activity from a representative sample. **B.** Average values and standard deviations of eight experiments with at least four independently transformed plants are shown. Assay conditions: 20 μg of total soluble protein for 30 min incubation period. Enzyme activity is presented as percent conversion of chloramphenicol to acetylchloramphenicol. Arrowhead indicates the position of 3-acetylchloramphenicol.

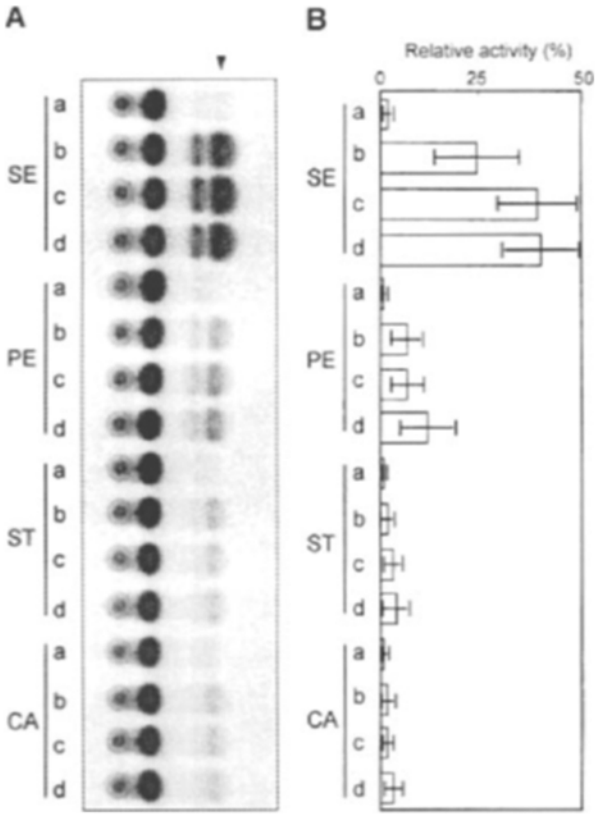

Figure 4. Induction of the PI-II promoter in flower by wounding, sucrose, and MJ. Sepals (SE), petals (PE), stamens (ST), and carpels (CA) were sampled before (a) or after wounding the tissue sections on MS medium (b), MS medium with 3% sucrose (c) or MS medium with 0.1 mM MJ (d) for 20 h. **A.** Analysis of CAT activity from a representative sample. **B.** Average values and standard deviations of 12 experiments with at least four independently transformed plants are shown. Assay conditions: 50 μg of total protein for 60 min incubation period. Enzyme activity is presented as percent conversion of chloramphenicol to acetylchloramphenicol. Arrowhead indicates the position of 3-acetylchloramphenicol.

observed (Fig. 4).

Transient Assay of PI-II Promoter

We established a transient assay system to examine the *cis*-elements that control expression and regulation of the PI-II promoter. Electroporation conditions were optimized with BY2 protoplasts and the Gene Pulser II electroporator. An electric pulse that was applied to the protoplasts without added DNA resulted in no detectable CAT activity. This indicated that the protoplasting and electric shock did not induce PI-II promoter activity (data not shown). However, the addition of 5 to 30 μg DNA significantly increased CAT activity. Interestingly, induction did not depend on the amount of DNA; 5 μg of DNA apparently was sufficient (data not shown).

Promoter activity was higher when capacitance was increased. However, when higher capacitances (>600 μF) were used, protoplasts were severely damaged and co-aggregated; 400 μF appeared to be optimum. The field strength of 0.75 kV/cm always gave higher CAT activity than 0.4 kV/cm (Fig. 5). As a control, the CaMV 35S promoter-*cat* fusion molecule was used for electroporation. Viability was measured by counting the regenerating protoplasts 48 h after electroporation. Cell numbers remained constant; results were similar for the entire experimental group.

The region between −611 and −500 of the PI-II promoter, which contained the wound response element (WRE), the sucrose response elements (SREs), and the MJ response element (MRE), was PCR-amplified and inserted into the XhoI/BglII site of pGA785.

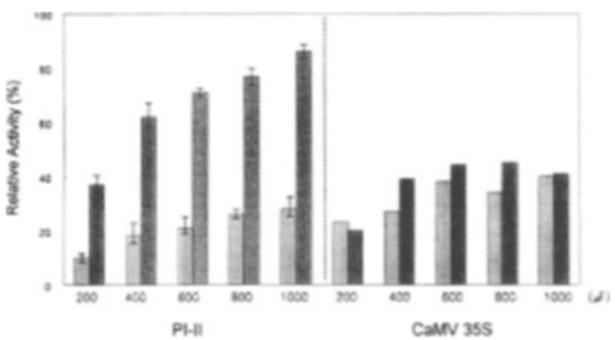

Figure 5. CAT activities of PI-II-*cat* and CaMV 35S-*cat* in transient assays. From 200 to 1000 μF of electrical capacitance were used for electroporation of BY-2 protoplasts at the field strength of 0.4 kV/cm (gray bar) and 0.75 kV/cm (black bar). CAT assay condition: PI-II-*cat*, 50 μg of total protein for 60 min incubation period; CaMV 35S-*cat*, 5 μg total protein for 60 min incubation period. For the PI-II promoter, data are the average values from three experiments.

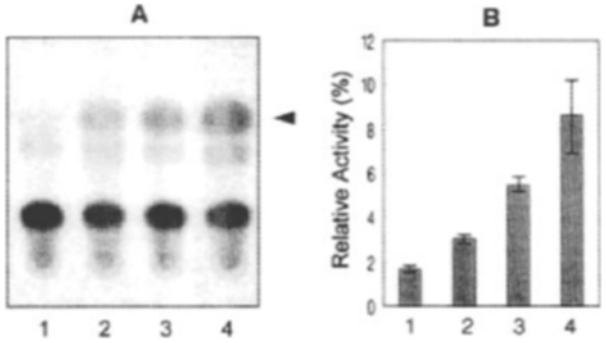

Figure 6. Effect of the upstream region between −611 and −500 of PI-II promoter in transient assay. Protoplasts of tobacco suspension cells were electroporated and harvested after incubating for 36 h. **A.** Analysis of CAT activity from a representative sample. Assay condition: 100 μg of total protein for 60 min incubation period. **B.** Average values of the relative CAT activities from three experiments. Lane 1, pGA785 (*nos*-101-*cat*-*6b* terminator); Lane 2, upstream region of PI-II promoter between −611 and −500 (−611~−500) fused to the *nos*-101 of pGA785; Lane 3, dimer of the (−611~−500) fused to the *nos*-101 of pGA785; Lane 4, trimer of the (−611~−500) fused to the *nos* −101 of pGA785. Enzyme activity is presented as percent conversion of chloramphenicol to acetylchloramphenicol. Arrowhead indicates the position of 3-acetylchloramphenicol.

Although the upstream region of the PI-II promoter showed low-level CAT activity, activity increased in proportion to the degree of multimerization in the −611/−500 region (Fig. 6).

DISCUSSION

We measured expression patterns of the PI-II promoter in various organs of transgenic tobacco plants at various developmental stages. The PI-II promoter activity was low at the seedling stage, but increased as plants developed. Eight weeks after germination, activity was higher in old leaves than in young leaves. These results suggest that the PI-II gene is temporally and spatially regulated.

The temporal and spatial regulation of the PI-II promoter is similar to that reported by An et al. (1988b) for the *nos* promoter, a well-characterized promoter from *Agrobacterium*. There, the *nos* promoter was highly active in cotyledons and old leaves, but only weakly expressed in young leaves However, *nos* promoter activity was high in roots, whereas our PI-II promoter activity was low there. At the flowering stage, the PI-II promoter activity also was low in both reproductive and vegetative organs. Young flowers had retained weak *nos* promoter activity, which

increased during flower development, and became highest at anthesis. Because the PI-II promoter in the current study was expressed weakly in floral buds, it would be interesting to examine whether PI-II activity also increases at anthesis.

The PI-II promoter was induced by wounding in leaves, stems, petioles, sepals, and petals, but was only weakly induced in roots, stamen, and carpels (Figs. 2 and 4). The molecular mechanism involved in wound induction of the PI-II promoter is apparently different from that of the *nos* promoter, because the latter was wound-inducible in a wide variety of organs (Keil et al., 1989; An et al., 1990). Wound induction of the PI-II promoter was further increased by sucrose treatment in leaves, petioles, and sepals. In stems and petioles, treatment with MJ resulted in higher levels of PI-II promoter activity than with sucrose. These differences among tissue types are not well understood, although several explanations might be proposed, including the possible presence of tissue-specific factor(s).

The upstream region of the PI-II promoter, between −611 and −500, contains wound, sucrose, and MJ response elements (Kim et al., 1992). We are now generating mutant promoters, which are replaced by a ClaI restriction site every 10 bp between −611 and −500, to analyze the *cis*-elements responsible for regulating the PI-II promoter.

Because of the slow process inherent to plant regeneration, *Agrobacterium*-mediated transformation methods require several weeks to several months to obtain stable transformants. Therefore, we established a transient assay system using electroporation to quickly analyze promoter elements. In this study, we concluded that a field strength of 0.75 kV/cm and 400 μF capacitance were optimal for the tobacco BY2 cells. This result contrasts somewhat with the parameters of 0.8-kV/cm field strength and 125-μF capacitance for *Nicotiana plumbaginifolia* leaf protoplasts, as specified by Bio-Rad in the Gene Pulser Electroprotocols. This discrepancy may be due to the difference in size of protoplasts or cellular contents.

Using the transient expression assay, we observed that the upstream region of the PI-II promoter (−611 and −500) exhibited low-level expression (Fig. 6). Promoter activity also increased, proportional to the degree of multimerization in the −611/−500 region. It is unclear whether this low expression resulted from inadequacy of the promoter itself or because of differences in other regions, e.g., terminator. CAT activity was shown to be significantly lower in the 3' deletion mutants compared with a wild-type promoter (Kim et al., 1991). This reduced promoter activity was probably due to the difference in the construction of chimaeric molecules. The mutant promoters were fused to the truncated *nos* promoter -101 and the *6b* terminator, which is about 50 times weaker than the PI-II terminator (An et al., 1989). The PI-II terminator region appears to enhance gene expression by increasing the mRNA half-life (Graham et al., 1986). Further studies, e.g., analysis of mutant promoters and an in-vitro binding assay, are necessary to define the exact *cis*- and *trans*-acting factors. By using transgenic tobacco plants that carry the 5'- and 3'- deletion mutation of the promoter, the *cis*-elements involved in expression for the developmental stages and organ types also could be identified.

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