The Plant Cell, Vol. 1, 115–122, January 1989, © 1989 American Society of Plant Physiologists

Functional Analysis of the 3′ Control Region of the Potato Wound-Inducible Proteinase Inhibitor II Gene

Gynheung An,[1] Amitava Mitra, Hong K. Choi, Michael A. Costa, Kyungsook An, Robert W. Thornburg,[2] and Clarence A. Ryan

Institute of Biological Chemistry, Washington State University, Pullman, Washington 99164-6340

Proteinase inhibitor genes are expressed strongly in specific plant tissues under both developmental and environmental regulation. We have studied the role of the 3′ control region of the potato proteinase inhibitor II gene (PI-II) that is inducible in leaves in response to herbivore attacks or other severe wounding. Comparison of the terminator from the PI-II gene with two different terminators from the *6b* and 7 genes, driven by a common PI-II promoter-*cat* fusion molecule, indicated that the PI-II terminator provided the most efficient expression of *cat*. The PI-II terminator also caused a significantly elevated *cat* gene expression driven by the cauliflower mosaic virus 35S promoter. The increase in the level of expression is probably not due to the presence of an enhancer element in the PI-II terminator region, but to *cis*-acting elements involved in mRNA processing or stability. Both transient and stable transformation analyses of the deletion mutants in the 3′-flanking sequence indicated that about a 100-base pair DNA fragment surrounding the polyadenylation site is essential for the efficient gene expression. This region seems to consist of several regulatory elements, including the conserved sequence, CGTGTCTT, which is located 9 bases downstream from the polyadenylation site. The elements appear to contribute to the increased stability of mRNAs containing the PI-II terminator.

INTRODUCTION

Wounding of leaves of various plants by herbivore attacks induces the expression of proteinase inhibitor genes in nearby leaf cells as well as in leaves many centimeters from the wound sites (Ryan, 1987). A putative wound factor, or factors, cumulatively called the proteinase inhibitor inducing factor, is thought to mediate the signaling system (Ryan, 1987). Oligouronides, fragments of plant cell wall pectinaceous materials, induce the proteinase inhibitor genes when supplied to excised leaves and are thought to be involved in the early events of the signaling process (Bishop et al., 1984). Members of two small wound-inducible gene families, called Inhibitor I and Inhibitor II, have been isolated from tomato and potato (Lee et al., 1986; Cleveland et al., 1987; Thornburg et al., 1987; Sanchez-Serrano et al., 1986). The wound-inducible control regions of one of these genes, the potato proteinase inhibitor II gene (PI-II), have been shown to be present in about 1 kb each of the 5′ and 3′ regions of the gene (Thornburg et al., 1987). When the 5′ region of the gene was fused with the open reading frame of the chloramphenicol acetyltransferase (*cat*) gene and terminated with either the 3′ region of the PI-II gene or by the *6b* terminator from the pTiA6 plasmid, chloramphenicol acetyltransferase (CAT) activity was expressed only when the PI-II terminator was present.

In this report we have investigated further the possible role of the 3′ control region of the PI-II gene by constructing a number of chimeric molecules with various terminators and promoters to establish the role of the PI-II 3′ region in controlling the gene expression. These experiments, together with a deletion analysis of the PI-II 3′ control region, indicate that the terminator region contains elements that contribute to the stability of the mRNA that are ended by the PI-II terminator.

RESULTS

Functional Analysis of the PI-II Terminator Region

A potato PI-II-*cat* fusion molecule containing about 1 kb each of the 5′ and 3′ control regions of the PI-II gene was wound-inducible in leaves of transgenic tobacco plants.

[1] To whom correspondence should be addressed.

[2] Current address: Department of Biochemistry and Biophysics, Iowa State University, Ames, IA 50011.

However, replacement of the PI-II terminator with a heterologous terminator from the *6b* gene reduced significantly the wound response (Thornburg et al., 1987). To examine whether the higher level of expression of the construct with the PI-II terminator was due to the presence of an enhancer element in the terminator region of the inhibitor gene, the 3′-flanking region was placed in normal and reverse orientations (pGA680 and pGA681) at the upstream region of the *nos* promoter, which was linked to a reporter *cat* gene and the *6b* terminator (pGA553). To reduce variations between individual transformants and maximize *cat* expression (R. Johnson and C. A. Ryan, manuscript in preparation), leaf sections from at least 10 independent plants were pooled and incubated in a Murashige-Skoog medium for 24 h (Figure 1). We have observed earlier that the *nos* promoter is expressed weakly in mature leaves, but the promoter activity is induced strongly by wounding (G. An, unpublished data). Placing the terminator region in either orientation upstream of the *nos* promoter did not influence significantly the CAT expression driven from the *nos* promoter. Transient assay of these molecules resulted in a similar expression level in tobacco protoplasts (data not shown). Therefore, it appears that the 3′ control region of the PI-II gene does not carry an enhancer element that functions upstream from the *nos* promoter.

The role of the PI-II terminator in influencing the *cat* expression driven by a strong promoter was studied. Three hybrid molecules carrying the common CaMV 35S promoter and the *cat* coding region with terminator sequences from (1) the 7 gene of pTiA6 (pGA663), (2) no plant terminator (pGA730), and (3) the PI-II gene (pGA724) were constructed (Figure 2). Wound inducibility of these gene fusions was examined by stably transferring the molecules into tobacco plants and the leaf slice induction assay (see "Methods"). The 3′ control region of the 7 gene provided a suitable terminator for driving CAT activity by the 35S promoter (Figure 2). The terminator-less molecule also expressed CAT activity, but at a significantly reduced level. Replacement of the 7 terminator with the 945-base pair (bp) RsaI-TaqI fragment carrying the 3′-flanking sequences of the inhibitor gene restored the CAT activity to an even higher level than that of the molecules with the 7 terminator. However, wounding the tobacco leaves carrying this molecule did not enhance the gene expression. Analyses of these molecules by the transient assay system showed an even more pronounced difference in *cat* expression (data not shown). The PI-II terminator was about 3 times stronger than the 7 terminator, and deletion of the terminator reduced gene expression significantly. These observations supported the previous data—that the 3′ control region of the PI-II gene does not contain a wound-inducible enhancer element but is an effective terminator.

To explore further the possibilities that the PI-II 3′ control region contains an efficient terminator, we have constructed molecules that carry the same PI-II promoter and *cat* coding region with the different 3′ control regions: (1) the PI-II terminator (pGA783), (2) the 7 terminator (pGA820), and (3) the *6b* terminator (pGA821). The molecules pGA783 and pGA821 are almost identical to the plasmids reported previously, pRT45 (strongly wound-inducible) and pRT50 (weakly wound-inducible) (Thornburg et al., 1987), except that the pGA molecules do not carry the DNA sequences derived from pUC13. Eight independent tobacco plants carrying either pGA783, pGA820, or pGA821 were regenerated, and wound-inducible CAT

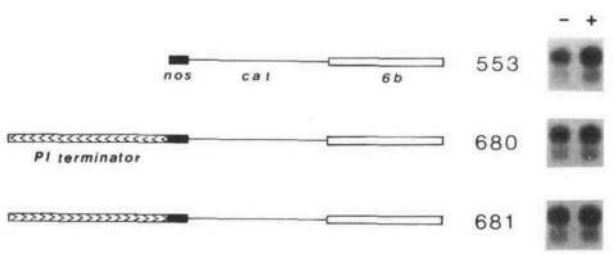

Figure 1. Effect of the PI-II Terminator on the *nos* Promoter Activity.

The 136-bp *nos* promoter fragment (−154 to −18), the 796-bp *cat* coding region, and the 732-bp SmaI-NaeI fragment of the pTiA6 transfer DNA (T-DNA) carrying the terminator sequence of the *6b* gene were derived from the plasmid 553-155 (An et al., 1986a). The 945-bp RsaI-TaqI fragment carrying the PI-II 3′ control region was placed in both orientations upstream of the *nos* promoter to generate pGA680 and pGA681. Autoradiograms of CAT activity before (−) and after (+) wounding tobacco leaf slices are shown at the right.

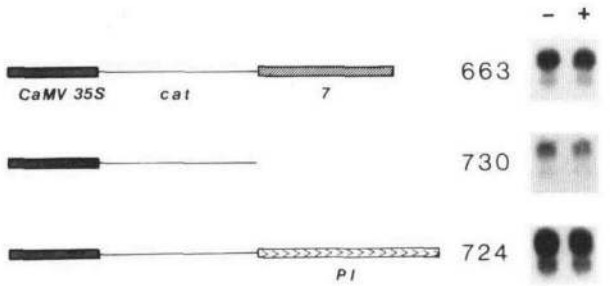

Figure 2. Effect of the PI-II Terminator on CAT Gene Expression Driven from the CaMV 35S Promoter.

The plasmid pGA663 contains the 419-bp CaMV 35S promoter fragment (HindII, the cap site), the 796-bp *cat* coding region, and the 743-bp ClaI-NdeI fragment of pTiA6 carrying the terminator region of the 7 gene (An et al., 1988a). The 3′-flanking region was replaced by the 945-bp PI-II 3′ terminator in pGA724. The right side shows autoradiograms of the CAT activity before (−) and after (+) wounding tobacco leaves carrying the chimeric molecule.

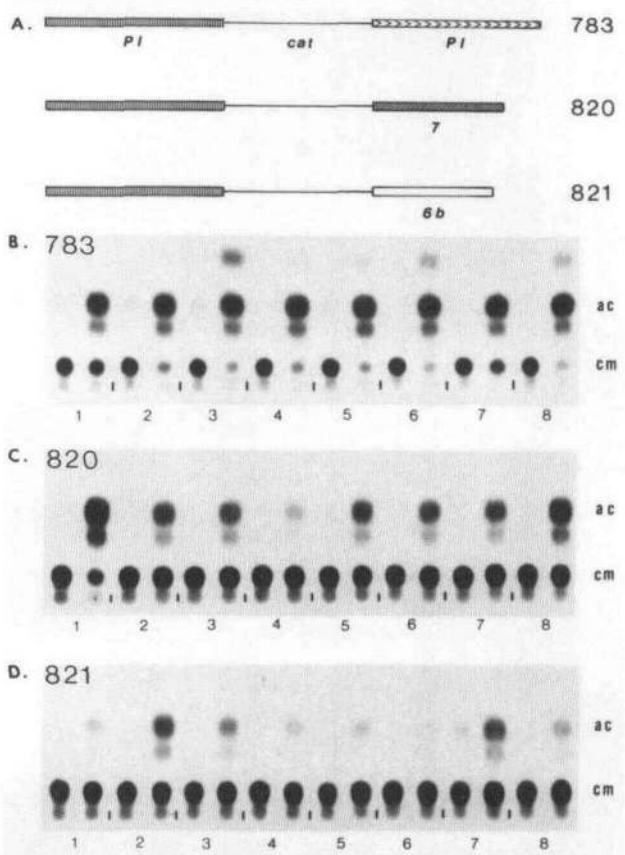

Figure 3. Effect of the PI-II Terminator on CAT Gene Expression Driven from the PI-II Promoter.

The structure of three different chimeric molecules carrying the common PI-II promoter (Thornburg et al., 1987), the *cat* coding region, and different terminators is shown in **(A)**. The terminator fragments were from the 945-bp PI-II 3′ control region (pGA783), the 743-bp 7 gene terminator (pGA820), and the 732-bp 6*b* gene terminator (pGA821). Autoradiograms represent the CAT activity of tobacco leaves carrying either pGA783 **(B)**, pGA820 **(C)**, or pGA821 **(D)**. The number indicates independently transformed tobacco plants. The CAT activities from both unwounded controls (left) and wounded leaf slices (right) are shown for each transformant. cm, chloramphenicol; ac, acetylchloramphenicol.

expression was measured from greenhouse-grown plants by the leaf-slice induction assay (Figure 3). All the transformed tobacco plants displayed wound-inducible CAT expression, suggesting that, regardless of the terminator sources, the PI-II promoter fragment alone is sufficient for the wound response. However, the wound-induced CAT activity was about 50 times greater in the plants carrying the PI-II terminator compared with those carrying the *6b* terminator. An intermediate level of the CAT expression was observed from the *7* terminator. Transient assay analysis of these molecules also demonstrated the differential expression of the *cat* reporter gene, depending on the terminator sequence. Similar effects of the terminators on the gene expression driven by the *nos* promoter were observed (data not shown). Therefore, it appears that the PI-II 3′ control region carries an efficient terminator that increases gene expression driven from its own as well as from heterologous promoters.

Deletion Analysis of the Terminator Region of the PI-II Gene

The 3′ *cis*-acting regulatory elements involved in efficient gene expression were identified by the deletion analysis of the PI-II terminator region. The 3′ control region was mutated by progressive deletions, and the DNA sequence of the terminator region and the end points of the deletion mutants were obtained (Figure 4). Effects of these mutations were examined by measuring the transient expression of the chimeric molecules that were introduced electroporatically into tobacco protoplasts. The results in Figure 5 demonstrate that the termination information

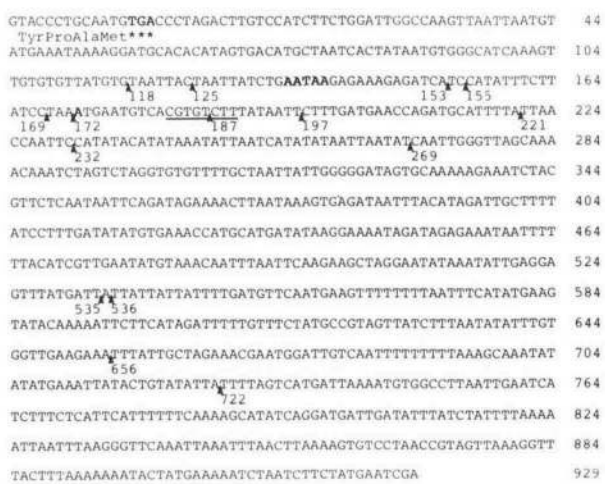

Figure 4. DNA Sequence of the PI-II 3′ Control Region and Deletion End Points.

The primary structure of the 945-bp RsaI-TaqI fragment carrying the PI-II 3′ control region was obtained by sequencing both strands using several deletion mutants generated by BAL-31 treatment (see "Methods"). Deletion end points that were used in this study are indicated below the DNA sequence with arrowheads. The numbers indicate distance from the termination codon. The termination codon TGA, polyadenylation signal AATAA at 135, and polyadenylation site at 172 are shown in boldface type. The conserved nucleotides, CGTGTCTT at 181, are underlined.

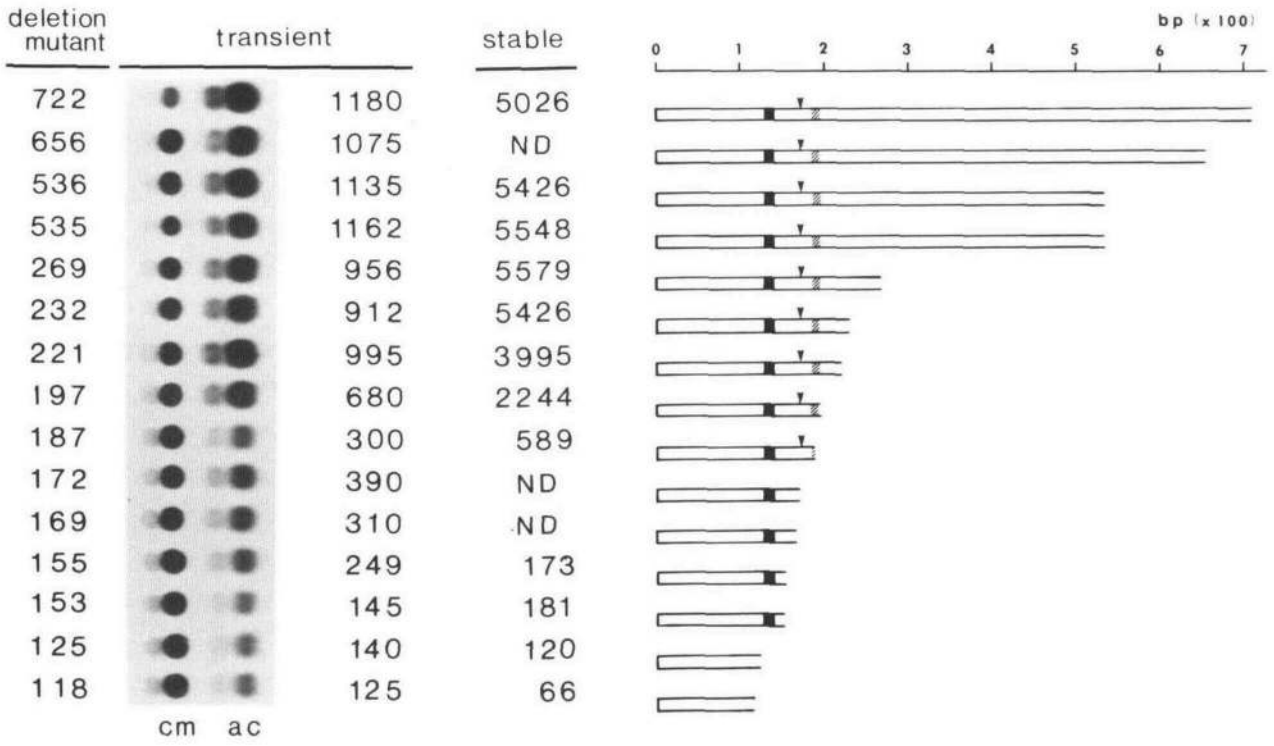

Figure 5. Deletion Analysis of the PI-II Terminator Region.

The end points of deletion mutants are indicated at the left. Schematic diagram of the deletion mutants is shown at the right. Solid boxes indicate the polyadenylation signal sequence AATAA, striped boxes represent the conserved sequence CGTGTCTT. The polyadenylation sites are shown with arrowheads. The CAT specific activities from both transient and stable transformation assays are presented as units per gram of protein. One unit of CAT catalyzes acetylation of 1 nmol of chloramphenicol/min at 37°C. ND, not determined; cm, chloramphenicol; ac, acetylchloramphenicol.

necessary for maximum gene expression is located within 220 bp from the terminator codon TGA. However, deletion of the 24-bp (between 197 and 220) and the 10-bp (between 187 and 196) DNA fragments located downstream from the polyadenylation site (Keil et al., 1986) reduced the CAT activity by a factor of three. Further deletions, including the polyadenylation site and the polyadenylation signal sequence AATAA, reduced the CAT expression to 10% of the wild-type activity. These results indicate that the DNA sequence surrounding the polyadenylation site is important for the efficient expression of the potato proteinase inhibitor II gene.

To examine whether the data obtained from the transient expression experiments indeed resulted from the wound-responsive induction of the inhibitor gene, tobacco plants were transformed stably with the *cat* chimeric molecules carrying the terminator mutants, and leaves from six independently transformed plants were examined for the wound-inducible gene expression. Very little CAT activity was observed in all the plants before wounding. However, almost all the plants accumulated CAT activity in wounded leaves. As observed from the transient assay system, the wound-inducible gene expression was high in the deletion mutants carrying more than 232 bp of the terminator sequences, and the deletion mutants 197 and 187 exhibited a significantly lower level of the CAT gene expression. Deletions of the DNA sequence adjacent to the polyadenylation site reduced the CAT expression further. The results from the stable transformation experiments are similar overall to that of transient expression, although the wild-type activity was much higher in the stable transformation assay. The results from both stable transformation experiments and transient assay systems suggest strongly that multiple regulatory elements that control the transcript level are present in the 100 bp of DNA fragments surrounding the polyadenylation site and that deletion of sequences further downstream does not influence the gene expression directly.

Analysis of Wound-Inducible mRNA Level in the Transgenic Plants Carrying the Deletion Mutants

To verify that results obtained from the CAT activity analysis represented the transcript level, the *cat*-specific mRNA level was measured before and after wounding leaves of a transformed tobacco plant exhibiting about an average level of CAT activity of each mutant. The results obtained by measuring the mRNA levels were almost identical to those obtained by measuring CAT activities (Figure 6).

DISCUSSION

The PI-II Terminator Region Enhances Gene Expression

The functional roles of the potato PI-II terminator were investigated in this study. Comparison of the PI-II terminator with the *7* terminator, *6b* terminator, or no plant terminator showed that expression of a reporter *cat* gene driven by either PI-II 5′ control region or the CaMV 35S promoter was most active when the PI-II terminator was used. It appears that the PI-II terminator region enhances gene expression by increasing the mRNA stability, in agreement with an earlier report that the PI-II mRNA has a long half-life (about 20 hr) (Graham et al., 1986).

It is unlikely that the lower levels of gene expression with either *7* or *6b* terminator resulted from deletion of the essential component of the terminators during the construction of chimeric molecules, since the cloned DNA fragments were long enough to cover the entire 3′ control region and a portion of the next gene (166-bp coding region of the *5* gene after the *7* terminator or 96-bp coding region of the *6a* gene after the *6b* terminator).

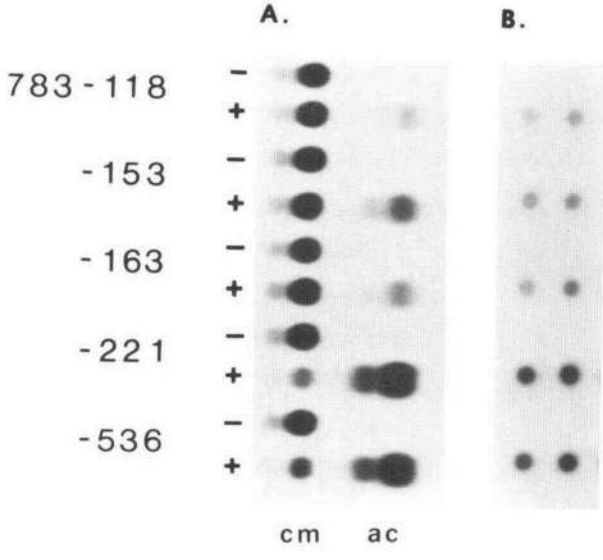

Figure 6. Comparison of mRNA Level and CAT Activity.

The end points of five deletion mutants are indicated on the left.
(A) CAT activities from control (−) and wounded leaf slices (+) are shown for each mutant.
(B) Dot-blot analysis of stable mRNA level.
Two (left) and 4 (right) μg of total RNAs were hybridized with the ^{32}P-labeled *cat* probe. cm, chloramphenicol; ac, acetylchloramphenicol.

The PI-II Terminator Consists of Multiple Regulatory Elements

We have demonstrated also that the PI-II 3′ region controlling efficient gene expression consists of multiple regulatory elements. The deletion analyses indicated that about 100 bp (between 118 and 221) surrounding the polyadenylation attachment site is essential for the terminator function. Removal of this sequence reduced gene expression by a factor of 10 in transient assay and 100 in the stable transformation system. It appears that several regulatory elements function coordinately to process mRNA, since progressive deletion of the region reduced the gene expression gradually.

cis-Acting elements located upstream and downstream from the polyadenylation site have been shown to be involved in endonucleolytic cleavage at a specific point in the 3′-untranslated region of mRNA, followed by the addition of several A residues to the cleaved end (Birnstiel et al., 1985). The consensus hexanucleotide AATAAA is located 10 to 30 bases upstream from the polyadenylation site in animals (Proudfoot and Brownlee, 1976) and plants (Joshi, 1987). Mutations in this signal resulted in abnormal and inefficient processing of the pre-mRNA (Higgs et al., 1983; Wickens and Stephenson, 1984). It has been suggested that the sequence is involved in selection of the cleavage site by associating with small nuclear RNA (Hashimoto and Steitz, 1986). In addition to the hexanucleotide, a 50-bp region located downstream from the polyadenylation site is required for efficient processing of animal mRNA (McDevitt et al., 1984; Gil and Proudfoot, 1984). This region includes a conserved sequence, YGTGTTYY. Comparison of DNA sequences near the polyadenylation site from 46 plant genes revealed a similar conserved sequence, YGTGTTTT (Joshi, 1987).

Despite these similarities, significant differences must exist in the terminator region between plants and animals since animal polyadenylation signals were utilized inefficiently in plant cells (Hunt et al., 1987). Extensive analyses of the plant terminator region would be necessary to evaluate whether these conserved sequences are also

involved in mRNA processing in plants. Our results indicate that the deletion mutant 232, which carries 59 bp downstream sequences from the polyadenylation site, exhibited full-strength gene expression in both stable and transient assay systems. Progressive deletion of this downstream region (Figure 5) reduced the transcript level gradually to a significantly lower level. Therefore, it appears that this 59-bp sequence is essential for the production of the mature mRNA from the PI-II gene and that the region contains several regulatory elements that function coordinately to yield the maximum processing. One such element is likely to be the conserved sequence CGTGTCTT, which is found 9 bp downstream from the polyadenylation site, since deletion of this sequence (mutant 187) affected the transcript level drastically. Although deletions of almost all the terminator sequence, including the polyadenylation site, resulted in significant reduction of gene expression, residual transcript levels were found in wounded leaves transformed with these mutants. This is probably due to a cryptic polyadenylation site present further upstream from the authentic polyadenylation site (A. Mitra and G. An, unpublished data).

Transient assays in electroporated cells provided a quick and convenient analysis of the PI-II promoter-driven gene expression. During the preparation of protoplasts, or the recovery period from the treatment, the wound-inducible factors necessary for the PI-II promoter activity must be activated and interact with the promoter. Although the fully induced level is lower in the transient assay system, the gene expression was strong enough to measure effects of deletion mutations. However, transient expressions appear to be controlled less stringently by regulatory factors as found in this study and in previous experiments (Ebert et al., 1987).

Conclusion

We have reported previously that the PI-II promoter driven *cat* gene was expressible in plants with the PI-II terminator but not with the *6b* terminator. In this study we have demonstrated that the chimeric molecule carrying the *6b* terminator is also wound-inducible by employing a more sensitive system, the leaf-slice induction assay.

Analysis of more than 300 independently transformed tobacco plants carrying a chimeric molecule consisting of the PI-II promoter and mutant terminator showed that, although the level of gene expression was variable, the chimeric gene expression was wound-inducible and leaf-specific in all the transgenic plants. Therefore, the *cis*-acting regulatory elements involved in wound response and tissue specificity are not influenced by the surrounding DNA sequences or by the chromatin structure.

We have presented evidence that the terminator region of the potato PI-II gene enhances expression of not only its own but also heterologous plant genes, probably by increasing mRNA stability, and that about a 100-bp DNA sequence surrounding the polyadenylation site is necessary for efficient gene expression. Such an efficient terminator may be useful to strongly express a foreign transcript in transgenic plants.

METHODS

Bacterial Strains and Plant Materials

Escherichia coli strain MC1000 (Casadaban and Cohen, 1980) was used as the host for the routine cloning experiments. *Agrobacterium tumefaciens* strain LBA4404 (Hoekema et al., 1983), carrying avirulent helper Ti plasmid pAL4404, was used for the transformation of tobacco (*Nicotiana tabacum* cv Xanthi) plants grown aseptically in MS medium (Murashige and Skoog, 1962).

Generation of Deletion Mutants

The deletion mutants were generated by BAL-31 treatment of the PI-II terminator fragment as described earlier (Ha and An, 1988). The 945-bp RsaI-TaqI fragment carrying the PI-II 3′ control region, along with the *cat* coding region that is under the control of the PI-II promoter, was cloned into the multiple cloning site of pGA617, which was derived from pUC19 by inserting an oligonucleotide carrying the sites for Asp718, XhoI, and StuI into the unique SspI site. The resulting plasmid pGA726 was opened at the unique SacI site located at the 3′ end of the PI-II terminator fragment and treated partially with an exonuclease, BAL-31. After digestion with StuI, the molecules were self-ligated. Size of deletions was determined by analyzing the structure of the mutated molecules, followed by sequencing the deletion end points (Maxam and Gilbert, 1977).

Transformation of Plants

The deleted molecules were cloned into a binary Ti plasmid vector, pGA628, which was derived from pGA472 (An et al., 1985). The plasmids were transferred into *A. tumefaciens* by the direct DNA transfer method (An et al., 1988b). Tobacco leaf slices were transformed stably by the cocultivation method as described earlier (Horsch et al., 1985; An et al., 1986b).

Transient Assay

Protoplasts prepared from an exponentially growing tobacco suspension culture were electroporated with 20 μg of plasmid DNA and 50 μg of carrier DNA using a 210-μF capacitor at 400 V across a 1-cm path (Fromm et al., 1985; Ebert et al., 1987). The protoplasts were incubated for 36 hr at 28°C and then harvested for the CAT assay.

Leaf-Slice Induction Assay

Greenhouse-grown tobacco leaves were washed several times with distilled water and cut to about 1-cm^2 sections. These leaf slices were placed in Murashige and Skoog liquid medium con-

taining 3% sucrose, 50 μg/ml kanamycin sulfate (Sigma), 200 μg/ml cefotaxime sodium (Hoechest-Roussel Pharmaceuticals), and 200 μg/ml carbenicillin disodium (Pfizer Inc.). The leaf slices were wounded by making several small holes in each section with forceps. The samples were incubated at 28°C for 24 hr under light (50 $\mu E/m^2/sec$).

Analysis of Gene Expression

CAT activity was measured by the TLC method using ^{14}C-chloramphenicol as described earlier (Gorman et al., 1982). RNA dot-blot hybridization was performed as reported previously (Mitra and An, 1989).

ACKNOWLEDGMENTS

This work was supported in part by grants from the National Science Foundation (DCB-8616018 to G.A. and DCB-8608594 to C.A.R.) and Enichem Americas Inc. (to C.A.R.).

Received October 26, 1988.

REFERENCES

An, G., Watson, B.D., Stachel, S., Gordon, M.P., and Nester, E.W. (1985). New cloning vehicles for transformation of higher plants. EMBO J. **4,** 277–284.

An, G., Ebert, P.R., Yi, B.-Y., and Choi, C. H. (1986a). Both TATA box and upstream regions are required for the nopaline synthase promoter activity in transformed tobacco cells. Mol. Gen. Genet. **203:** 245–250.

An, G., Watson, B.D., and Chiang, C.C. (1986b). Transformation of tobacco, tomato, potato, and *Arabidopsis thaliana* using a binary vector system. Plant Physiol. **81,** 301–305.

An, G., Costa, M.A., Mitra, A., Ha, S.B., and Marton, L. (1988a). Organ-specific and developmental regulation of the nopaline synthase promoter in transgenic tobacco plants. Plant Physiol. **88,** 547–552.

An, G., Ebert, P.R., Mitra, A., and Ha, S.B. (1988b). Binary vectors. In Plant Molecular Biology Manual, S.B. Gelvin and R.A. Schilperoort, eds (Dordrecht, The Netherlands: Martinus Nijhoff), pp. A3, 1–19.

Birnstiel, M.L., Busslinger, M., and Strub, K. (1985). Transcription termination and 3′ processing: The end is in site! Cell **41,** 349–359.

Bishop, P.D., Pearce, G., Bryant, J.E., and Ryan, C.A. (1984). Isolation and characterization of the proteinase inhibitor-inducing factor from tomato leaves: Identity and activity of poly- and oligo-galacturonide fragments. J. Biol. Chem. **259,** 13172–13177.

Casadaban, M.J., and Cohen, S.N. (1980). Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*. J. Mol. Biol. **138,** 179–207.

Cleveland, T.E., Thornburg, R.W., and Ryan, C.A. (1987). Molecular characterization of a wound-inducible inhibitor I gene from potato and the processing of its mRNA and protein. Plant Mol. Biol. **8,** 199–207.

Ebert, P.R., Ha, S.B., and An, G. (1987). Identification of an essential upstream element in the nopaline synthase promoter by stable and transient assays. Proc. Natl. Acad. Sci. USA **84,** 5745–5749.

Fromm, M.E., Taylor, L.P., and Walbot, V. (1985). Expression of genes electroporated into monocot and dicot plant cells. Proc. Natl. Acad. Sci. USA **82,** 5824–5828.

Gil, A., and Proudfoot, N.J. (1984). A sequence downstream of AAUAAA is required for rabbit β-globin mRNA 3′-end formation. Nature **312,** 473–474.

Gorman, C.M., Moffat, L.F., and Howard, B.H. (1982). Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2,** 1044–1051.

Graham, J.S., Hall G., Pearce, G. and Ryan, C.A. (1986). Regulation of synthesis of proteinase inhibitors I and II mRNAs in leaves of wounded tomato plants. Planta (Berl.) **169,** 399–405.

Ha, S.B., and An, G. (1988). Identification of upstream regulatory elements involved in the developmental expression of the *Arabidopsis thaliana cab1* gene. Proc. Natl. Acad. Sci. USA **85,** 8017–8021.

Hashimoto, C., and Steitz, J.A. (1986). A small nuclear ribonucleoprotein associates with the AAUAAA polyadenylation signal in vitro. Cell. **45,** 581–591.

Higgs, D.R., Goodbourn, S.E.Y., Lamb, J., Clegg, J.B., and Weatherall, D.J. (1983). β-Thalassaemia caused by a polyadenylation signal mutation. Nature (Lond.) **24,** 398–400.

Hoekema, A., Hirsch, P.R., Hooykaas, P.J.J., and Schilperoort, R.A. (1983). A binary plant vector strategy based on separation of *vir*-and T-region of the *Agrobacterium tumefaciens* Ti-plasmid. Nature (Lond.) **303,** 179–181.

Horsch, R.B., Fry, J.E., Hoffmann, N.L., Eichholtz, D., Rogers, S.G., and Fraley, R.T. (1985). A simple and general method for transferring genes into plants. Science **227,** 1229–1231.

Hunt, A.G., Chu, N.M., Odell, J.T., Nagy, F., and Chua, N.-H. (1987). Plant cells do not properly recognize animal polyadenylation signals. Plant Mol. Biol. **8,** 23–35.

Joshi, P.C. (1987). Putative polyadenylation signals in nuclear genes of higher plants: A compilation and analysis. Nucleic Acids Res. **15,** 9627–9640.

Keil, M., Sanchez-Serrano, J., Schell, J. and Willmitzer, L. (1986). Primary structure of a proteinase inhibitor II gene from potato (*Solanum tuberosum*). Nucleic Acids Res. **14,** 5641–5650.

Lee, J.S., Brown, W.E., Graham, J.S., Pearce, G., Fox, E.A., Dheher, T.W., Ahern, K.G., Pearson, G.D., and Ryan, C.A. (1986). Molecular characterization and phylogenetic studies of a wound-inducible proteinase inhibitor I gene in *Lycopersicon* species. Proc. Natl. Acad. Sci. USA **83,** 7277–7281.

Maxam, A.M., and Gilbert, W. (1977). A new method for sequencing DNA. Proc. Natl. Acad. Sci. USA **74,** 560–564.

McDevitt, M.A., Imperiale, M. J., Ali, H., and Nevins, J. (1984). Requirement of a downstream sequence for generation of a

poly(A) addition site. Cell **37,** 993–999.

Mitra, A., and An, G. (1989). Three distinct regulatory elements comprise the upstream promoter region of nopaline synthase gene. Mol. Gen. Genet., in press.

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant. **15,** 473–497.

Proudfoot, N.J., and Brownlee, G.G. (1976). 3′-Noncoding region sequences in eukaryotic messenger RNA. Nature **263,** 211–214.

Ryan, C.A. (1987). Oligosaccharide signalling in plants. Annu. Rev. Cell Biol. **3,** 295–317.

Sanchez-Serrano, J., Schmidt, R., Schell, J., and Willmitzer, L. (1986). Nucleotide sequence of proteinase inhibitor II encoding cDNA of potato (Solanum tuberosum) and its mode of expression. Mol. Gen. Genet. **203,** 15–30.

Thornburg, R.W., An, G., Cleveland, T.E., Johnson, R., and Ryan, C.A. (1987). Wound-inducible expression of a potato inhibitor II-chloramphenicol acetyltransferase gene fusion in transgenic tobacco plants. Proc. Natl. Acad. Sci. USA **84,** 744–748.

Wickens, M., and Stephenson, P. (1984). Role of the conserved AAUAAA sequence: Four AAUAAA point mutants prevent messenger RNA 3′ end formation. Science **226,** 1945–1951.