Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 603–607, January 1990
Botany

Wound-inducible nuclear protein binds DNA fragments that regulate a proteinase inhibitor II gene from potato

(deletion analysis/gel retardation/elicitor-induced gene/transformed tobacco)

CURTIS J. PALM, MICHAEL A. COSTA, GYNHEUNG AN, AND CLARENCE A. RYAN*

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

Contributed by Clarence A. Ryan, October 10, 1989

ABSTRACT **Deletion analysis from the 3′ to the 5′ end of the promoter region of the wound-inducible potato proteinase inhibitor IIK gene has identified a 421-base sequence at −136 to −557 that is necessary for expression. Utilizing DNA band-shift assays, a 10-base sequence within the 421-base region was found to bind a nuclear protein from wounded tomato leaves. This 10-base sequence is adjacent to an 8-base consensus sequence at −147 to −155 that is present in the promoter region of several elicitor-inducible genes from various other plants. The evidence suggests that a complex set of cis- and trans-acting elements within the −136 to −165 region of the potato IIK gene may be involved with the signaling mechanisms that regulate the inducibility of this gene in response to pest and pathogen attacks.**

Two nonhomologous proteinase inhibitors, inhibitor I and inhibitor II, are induced by wounding in both potato and tomato plants (1, 2). The induction of the inhibitors in response to wounding is systemic, as both inhibitors are synthesized in unwounded as well as in wounded leaves (3, 4). Synthesis of the inhibitor proteins and mRNAs in response to wounding is regulated at the level of transcription (5). Genes encoding the proteinase inhibitor I and II proteins have been isolated from both potato (6, 7) and tomato (8) and a chimeric gene containing the 5′ promoter region of the potato proteinase inhibitor IIK gene, fused to the reporter chloramphenicol acetyltransferase (CAT) gene, has been demonstrated to be wound inducible in transgenic tobacco plants (6, 9).

The mechanism by which the plant can signal proteinase inhibitor gene expression is not well defined. Oligogalacturonides, isolated from tomato leaf cell walls, can induce the expression of the proteinase inhibitor genes in leaves in the absence of severe wounding when supplied through cut petioles of excised plants (10). These oligogalacturonides, cumulatively known as proteinase inhibitor-inducing factor, are thought to be released from wounded tissues as early signals in the pathway that ultimately leads to both localized and systemic wound-induced expression of the proteinase inhibitor genes (11). The uronides, however, may not be the systemic signal, since they probably do not move systemically through the plant (12). This implies that a second systemic signal must be regulating the systemic response (13).

In this report we have used deletion analysis in stably transformed plants to identify wound-inducible cis elements of the 5′ region of the inhibitor IIK gene. To identify wound-inducible trans-acting proteins that might regulate these elements, we have used gel-retardation assay with fragments of the promoter region of the inhibitor IIK wound-inducible gene and nuclear extracts from wounded and unwounded tomato leaves. We have identified a sequence in the promoter region of the gene that is necessary for gene expression and that also binds a wound-inducible nuclear protein†.

MATERIALS AND METHODS

Bacterial Strains and Plant Materials. *Escherichia coli* strain MC1000 (14) was used as the host for routine cloning experiments. *Agrobacterium tumefaciens* strain LBA4404 (15), carrying avirulent helper Ti plasmid pAL4404, was used for the transformation of tobacco (*Nicotiana tabacum* cv. Xanthi). Plants used for stable transformation were grown aseptically in MS medium (16).

Generation of Deletion Mutants. A 923-bp *Taq* I/*Sca* I fragment carrying the proteinase inhibitor II 5′ control region was ligated with a truncated nopaline synthase (*nos*) promoter −101 (17) that was followed by the coding region of the CAT gene and the terminator of the transcript *6b* gene (17). The 600 bp of stuffer sequence isolated from pUC plasmid was inserted between the proteinase inhibitor II and *nos* promoter to facilitate generation of deletion mutants. The resulting plasmid pGA788 was opened at the unique *Pst* I site located at the 3′ end of the proteinase inhibitor II promoter and treated partially with exonuclease BAL-31. The molecules were cut at the unique *Stu* I site in front of the *nos* −101. Self-ligation of these molecules removed the 600-bp pUC sequence and generated a set of 3′ deletion mutants of the proteinase inhibitor II promoter, which were fused to the truncated *nos* promoter. These plasmids were named pGA814-xxx, where xxx is the deletion end point of each mutant.

Transformation of Plants. The mutant plasmids, pGA814-xxx, were cloned into a binary Ti plasmid vector, pGA628, which is a derivative of pGA492 (18), with *Hin*dIII enzyme. The plasmids were transferred into *A. tumefaciens* by the direct DNA transfer method (19). Tobacco leaf slices were transformed stably by the cocultivation method as described (19). Leaves from the regenerated transformants were wounded and CAT activity was measured by the TLC method using [^{14}C]chloramphenicol as described (19).

Nuclei Isolation and Protein Extraction. Tomato plants at 18–21 days (var. castlemart) were wounded three times at 1-hr intervals. Plants were harvested 6–8 hr from initial wounding. Nongreen stem and root tissue from wounded plants was also collected.

Nuclei were isolated by a method modified from ref. 20. Plant tissue was ground to a fine powder in liquid nitrogen. Nuclear extraction buffer [NEB; 10 mM Mes, pH 5.2/0.25 M sucrose/10 mM NaCl/5 mM NaF/5 mM EDTA/2% dextran

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: CAT, chloramphenicol acetyltransferase; PMSF, phenylmethylsulfonyl fluoride.

*To whom reprint requests should be addressed.

†The sequence reported in this paper has been deposited in the GenBank data base (accession no. M29965).

T-40/0.22 mM spermine tetrahydrochloride/0.6 mM dispermidine triphosphate/22 mM 2-mercaptoethanol/0.024% Triton X-100/0.2 mM phenylmethylsulfonyl fluoride (PMSF)/0.1% bovine serum albumin fraction V/leupeptin, pepstatin A, chymostatin, and antipain (0.5 μg/ml each)] was added (4 ml per g of tissue) and the suspension was stirred at 4°C for 30 min. The suspension was then homogenized with a polytron twice (setting 5) for 30 sec each. The suspensions were filtered successively through cheesecloth and then 300-μm, 100-μm, and 53-μm nylon mesh. The filtrate was centrifuged at 1300 × *g* for 20 min in a Sorvall HB1000 centrifuge. The pellet was resuspended in 50 ml of NEB and transferred to a 50-ml tube and pelleted again. The pellet was resuspended in 20 ml of NEB and 30 ml of NEB/Percoll [NEB in 80% Percoll (vol/vol) without dextran T-40 and 0.004% Triton X-100] was added. The suspension was mixed by inversion and the nuclei were pelleted by centrifugation at 1300 × *g* for 30 min. Nuclear proteins were prepared from the crude nuclei pellet essentially as described by Dignam *et al.* (21) except the extraction buffer contained 20 mM Hepes (pH 7.9), 25% (vol/vol) glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 M NaCl, 0.5 mM PMSF, 0.5 mM dithiothreitol, and leupeptin, pepstatin A, chymostatin, and antipain (0.5 μg/ml each), and the dialysis buffer [20 mM Hepes (pH 7.9), 20% (vol/vol) glycerol, 0.2 mM EDTA, 20 mM NaF, 0.5 mM PMSF, and 0.5 mM dithiothreitol]. Dialyzed nuclear extracts were aliquoted and frozen in liquid nitrogen and stored at −80°C. The nuclear extracts were standardized by the Bradford protein assay (32) and this was found to correlate well with nucleic acid measurements (A_{260}) and nuclei counting.

DNA Band-Shift Assays. DNA binding proteins were identified by the gel electrophoresis mobility retardation assay (22, 23). DNA fragments used in the binding assays were isolated from agarose gels using DEAE-cellulose membrane (NA45; Scheicher and Schuell) (24). DNA fragments were radioactively labeled by end-filling reaction with [^{32}P]dCTP (New England Nuclear) using the Klenow fragment of *E. coli* DNA polymerase (25). Binding reactions were performed in 20-μl volumes. Standard reaction mixtures contained 20 mM Tris·HCl (pH 8.0), 3% (vol/vol) glycerol, 0.5 mM dithiothreitol, 0.25 mM PMSF, 1 mM EDTA, 50 mM NaF, 4 μg of poly(dI-dC)·poly(dI-dC) (Pharmacia), 15 μg of protein extract, and 0.1–1 ng of end-labeled DNA fragment. After 10 min of incubation at room temperature, binding mixtures were separated by electrophoresis in 4% polyacrylamide gels with 0.5× TBE (45 mM Tris·HCl/45 mM boric acid/1 mM EDTA) as the gel and running buffer. Gels were dried and exposed to film to visualize bands. When applicable, free and bound bands were quantified using an LKB 2202 Ultrascan laser densitometer.

RESULTS AND DISCUSSION

Deletion Analysis of the 5′ Region of the Proteinase Inhibitor II 5′ Control Region. Previous research had demonstrated that the element responsible for the wound-inducible expression of the potato proteinase inhibitor IIK gene is located in a 923-bp *Taq* I/*Sca* I fragment (Fig. 1) of the 5′ control region (9). To identify the cis-acting regulatory elements controlling expression of the proteinase inhibitor IIK gene, plasmid pGA788 carrying the 923-bp fragment of the proteinase inhibitor IIK promoter and the truncated *nos* promoter −101 was constructed. The 3′ end of the proteinase inhibitor IIK promoter was subjected to deletion mutagenesis as described in *Materials and Methods*. The resulting plasmids carried a fused promoter consisting of the proteinase inhibitor II deletion mutants and the *nos* −101, which was connected to a reporter CAT gene (Fig. 2*A*). The *nos* −101 promoter alone is silent but it becomes active when fused to a functional upstream regulatory element and the characteristics of the hybrid promoter are similar to the promoter that provided the upstream element (26). Therefore, we expected that the functional hybrids would be wound inducible. Fig. 1 shows the nucleotide sequence of the proteinase inhibitor IIK 5′ control region and the end points of five deletion mutants used for this study. Six to 12 independent transgenic tobacco plants carrying each deletion mutant were generated to reduce a potential variation due to the insertion position. Effects of deletions were studied by measuring expression of the CAT reporter gene before and after wound induction either in the presence or absence of sucrose. Sucrose enhances expression of the proteinase inhibitor II gene in potato leaves and transformed tobacco (R. Johnson and C.A.R., unpublished data). Results in Fig. 2*B* demonstrate that transgenic tobacco plants carrying the 3′ deletions −25, −48, and −135 exhibited wound-inducible CAT activity and the induction was further enhanced by sucrose. However, the deletion mutants, −557 and −717, did not respond to the wound

FIG. 1. Nucleotide sequence of the potato proteinase inhibitor IIK promoter region. Restriction enzyme sites used for the isolation of fragments for the binding studies are in boldface type. The 3′ deletion end points used for this experiment are indicated below the sequence with arrowheads. The numbers indicate relative position to the transcription initiation site, +1. Single and double underlines show positions of putative systemic wound-inducible sequence and uronide responsive sequence, respectively.

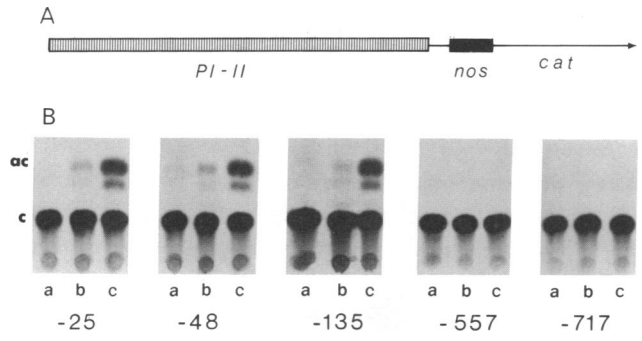

FIG. 2. Deletion analysis of the proteinase inhibitor IIK promoter. (*A*) Schematic diagram of the hybrid promoter between the inhibitor I 5′ control region and the *nos* promoter −101 that were connected to the reporter molecule CAT gene. (*B*) Wound induction of the hybrid promoter. Transgenic tobacco leaf was assayed before (lanes a) and after wounding without (lanes b) or with (lanes c) 3% sucrose. Among several transgenic plants, only results for a representative plant for each deletion are shown. c, Chloramphenicol; ac, acetylchloramphenicol.

induction, suggesting that within the 421 nucleotides between −136 and −557 is located a portion or an entire sequence of a regulatory element involved in the wound-inducible expression of the proteinase inhibitor IIK promoter.

Binding of Nuclear Proteins to the Inhibitor IIK Promoter. Five adjacent nonoverlapping DNA restriction fragments (Fig. 3*a* A–E), which together span the first 923 bp of the inhibitor IIK 5′ region, were isolated from pRT24 (6) by restriction enzyme digestion and purification by agarose gel electrophoresis. To isolate the fragments, pRT24 was digested with restriction enzymes that have sites at the borders of the fragments as shown in Fig. 3. In addition, for fragments A and E the plasmid was digested with *Bam*HI or *Pst* I, respectively. These enzymes have sites in the polylinker flanking the insert in pRT24 and allow for isolation of fragments A and E. If necessary, overhanging ends were filled in and the fragments were ligated individually into the *Hin*cII, *Bam*HI/*Hin*cII (for fragment A) or *Hin*cII/*Pst* I site (for fragment E) or pUC18. For binding assays, these recombinant plasmids were digested with *Eco*RI and *Hin*dIII and the promoter fragments were isolated from agarose gels. The polylinker sequences contained in these fragments did not alone show specific binding with nuclear extracts (data not shown). In addition, DNA fragments isolated from the deletion clones pGA814-135 and pGA814-165 were analyzed for their abilities to bind tomato leaf nuclear proteins. The two fragments F and G (Fig. 3*a*) differ only by an additional 30 bp at the 3′ terminus of fragment F. The seven DNA fragments were end labeled with ^{32}P and were used to seek DNA binding proteins that were present in nuclear extracts prepared from wounded tomato leaf tissue.

In band-shift assays, fragments A, B, E, and F exhibited retarded bands (Fig. 3*b*), indicating that they bind to nuclear proteins. The electrophoretic band shifts were eliminated by either proteinase or heat pretreatment of the extract (data not shown), providing evidence that the band shifts were caused by a protein–DNA interaction. In the binding assays in Fig. 3, a band shift is seen when fragment F but not fragment G is used in the assay, indicating that a nuclear protein binds in the −136 to −165 region. Because this sequence is within the region necessary for wound inducibility, we decided to focus on the characterization of the DNA–protein interaction from this area of the promoter.

Competition for Binding with Unlabeled DNA Fragments. To better define the sequence needed for binding, unlabeled DNA was added as a competitor to the binding reaction mixtures containing either labeled fragment B or F. A 100-

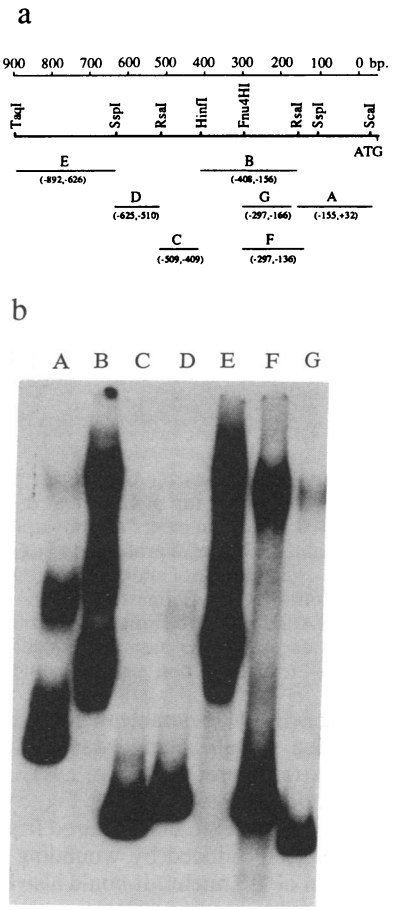

FIG. 3. Nuclear protein binding to 5′ flanking region of the proteinase inhibitor II gene. (*a*) Restriction endonuclease site map of potato proteinase inhibitor II gene 5′ flanking region. Numbers above map are bp. Zero coordinate was chosen as the transcription start site. ATG indicates the translation start site. Fragments isolated and used in the binding assays are indicated by solid lines below the map. End points of fragments are shown below fragments. Fragment sizes (in bp, including polylinker sequences) are as follows: A, 240; B, 300; C, 150; D, 165; E, 310; F, 160; G, 130. (*b*) Binding assays. Assays were as described in *Materials and Methods*; 1.3 fmol (0.12–0.25 ng depending on size) of ^{32}P-labeled fragment and 15 μg of nuclear extract were used in each reaction. Fragment used in each reaction is indicated above the lane.

fold excess of nonspecific competitors λ, *E. coli*, and salmon sperm DNA has no effect on the band shift seen with labeled fragment F (Fig. 4*a*). Fragments from the promoter region were also used as competitors in the binding reactions. Of the fragments used, only unlabeled fragments B and F were able to compete for the binding of the factor that binds to fragment F (Fig. 4*b*). The same two fragments competed for binding of the factor to fragment B (Fig. 4*c*), while excess amounts of fragments A, D, or G did not affect the binding of the factor with either fragment B or F. Thus, the binding site recognized by the factor is present on fragments B and F, but not on fragments A, D, or G.

Tissue Specificity of Binding Interaction. Nuclear extracts prepared from different tissues of the tomato plant were assayed for presence of the binding factor using fragment B (Fig. 5). The specific binding activity in extracts from wounded leaf was not detected in extracts made from stem or root tissue; however, a different binding activity was observed in root extracts. It is not known whether this root-specific binding activity recognizes the same or a different sequence on the fragment.

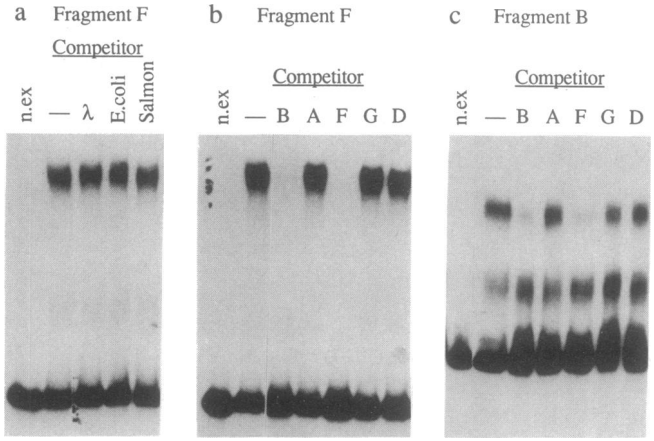

FIG. 4. Competition for DNA binding factor. ^{32}P-labeled fragments F and B (as indicated at top of figure) were used in binding assays with unlabeled DNA fragments as shown above the lanes. Binding assays contained 1.3 fmol of probe and 15 μg of extract. n.ex, No extract added; −, no competitor added. (*a*) A 100-fold excess (wt basis) of each competitor. λ, *Hin*fI digested λ DNA; *E. coli*, sheared *E. coli* genomic DNA; salmon, *Hin*fI digested salmon sperm DNA. (*b* and *c*) A 20-fold excess (molar basis) of the different unlabeled fragments was added to the reaction mixture as indicated.

Protein binding activity detectable in nuclear extracts from leaves of wounded tomato plants was always 2–5 times greater than in extracts prepared from nuclei isolated from leaves of unwounded plants (e.g., see Fig. 5). The binding factor present in nuclear extracts prepared from unwounded plants may have been induced by wounding that occurred during preparation of the nuclei. It could also be a precursor of the wound-inducible binding protein or a cofactor for the response. Purification of the factor should resolve this question.

Correlation of Deletion Analysis with DNA–Protein Binding Assays. Both the deletion analyses and gel retardation assays indicated that the region of the gene from −136 through −557 contains elements that regulate aspects of wound inducibility of the proteinase inhibitor IIK gene. The combination of results from the two analyses indicates that at least one of the controlling elements is located between −135 and −165.

The gel-retardation assays indicated that a protein factor is recognizing a specific sequence common to both fragments B and F that is not present in either fragment G or A (Fig. 6), and that at least part of this factor is wound inducible. The sequence that meets this requirement is −156 to −165. At least a portion of these 10 bp, AAGCGTAAGT, is essential for binding, but the complete sequence could include some flanking bases. The 10-bp region that is implicated in binding the wound-inducible factor is directly adjacent to the sequence ACCTTGCC (Fig. 6), that differs by only 1 base to a sequence identified by *in vivo* footprinting (27) as an elicitor-inducible motif. This motif is also present in the 5′ region of several other elicitor- or light-inducible genes (Table 1; ref. 27). This leads to the speculation that wound-inducible trans factors may be different than elicitor-induced trans factors.

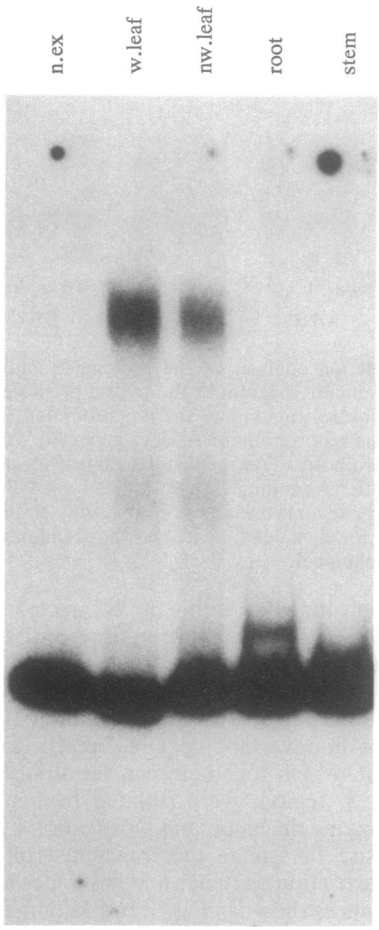

FIG. 5. Binding activity of nuclear extracts from different tomato tissues. Assays were performed as described in *Materials and Methods*. Probe for assays was fragment B. Nuclear extracts used in assays are from tissues indicated above figure. n.ex, No extract; w., wounded; nw., leaves from unwounded plants; root, root tissue from wounded plants; stem, lower stem tissue from wounded plants; 4.5 μg of protein was used in the assay mixtures containing leaf extracts and 9 μg of protein was used in the assay mixtures containing stem and root extracts.

Proteinase inhibitors that are systemically induced in plants are probably induced via a systemic signal that is not an oligosaccharide (12). It is possible that localized induction of proteinase inhibitors may be regulated by oligouronides (elicitors), whereas wounding may release an entirely different signal that is rapidly transported throughout the plants, inducing proteinase inhibitor synthesis via a different mechanism. Thus, the motif at −156 to −165 may bind a nuclear factor generated by a noncarbohydrate systemic response, while the elicitor-like motif may be regulated by the uronides and chitosans that activate localized expression of other defensive genes as well. Further analysis of this system should be of considerable value in helping to sort out the apparently complex mechanism that underlies the localized

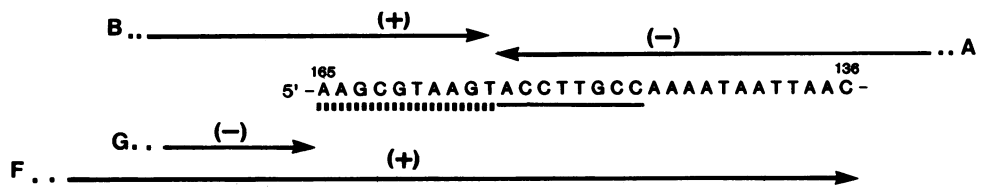

FIG. 6. The 30-base region identified by deletion and protein binding analyses to be important for expression. Shown above and below sequence are the regions contained in fragments A, B, F, and G. The region underlined by the dotted line is necessary for binding a wound-inducible nuclear protein. The region underlined with a solid line is the elicitor-responsive motif in Table 1.

Table 1. Conserved nucleotide sequence in oligouronide and UV light-inducible promoters

Sequence	Position	Source	Ref.
ACCTTGCC	−148	Potato PI-IIK	This report
ACCTA CC	−57	Parsley PAL-1	27
ACCTA CC	−52	Snapdragon CHS	28
AGCTA CC	−56	Corn C2	27
ACCTAACC	−53	Corn CHS2	27
ACCAACCC	−152	Parsley 4CL	29
ACCTA CC	−67	*Arabidopsis* CHS	30
ACCTAACC	−132	Parsley CHS	31

PI-IIK, proteinase inhibitor IIK; PAL, phenylalanine ammonia lyase; CHS and C2, chalcone synthase; 4CL, 4 coumerate:CoA ligase.

and systemic regulation of proteinase inhibitor genes in response to pest and pathogen attacks.

We thank Mike Lawton for helpful discussions on nuclei purification and Greg Wichelns for growing the plants. This work was supported in part by the Washington State University College of Agriculture and Home Economics Project 1791, grants from the United States Department of Agriculture (to G.A.), and the National Science Foundation (DCB8616018 to G.A. and DCB8608594 to C.A.R.), and a grant from EniChem Americas, Inc. (to C.A.R.).

1. Green, T. R. & Ryan, C. A. (1972) *Science* **175,** 776–777.
2. Green, T. R. & Ryan, C. A. (1973) *Plant Physiol.* **51,** 19–21.
3. Plunkett, G., Senear, D. F., Zuroske, G. & Ryan, C. A. (1982) *Arch. Biochem. Biophys.* **213,** 463–472.
4. Ryan, C. A. & An, G. (1988) *Plant Cell Environ.* **11,** 345–349.
5. Graham, J., Hall, G., Pearce, G. & Ryan, C. A. (1986) *Planta* **169,** 399–405.
6. Thornburg, R. W., An, G., Cleveland, T. E., Johnson, R. & Ryan, C. A. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 744–748.
7. Keil, M., Sanchez-Serrano, J., Schell, J. & Willmitzer, L. (1986) *Nucleic Acids Res.* **14,** 5641–5650.
8. Lee, J. S., Brown, W. E., Graham, J. S., Pearce, G., Fox, E. A., Dreher, T. W., Ahern, K. G., Pearson, G. D. & Ryan, C. A. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 7277–7281.
9. An, G., Mitra, A., Choi, H. K., Costa, M. A., An, K., Thornburg, R. W. & Ryan, C. A. (1989) *Plant Cell* **1,** 115–122.
10. Bishop, P. D., Pearce, G., Bryant, J. E. & Ryan, C. A. (1984) *J. Biol. Chem.* **259,** 13172–13177.
11. Ryan, C. A. (1987) *Annu. Rev. Cell. Biol.* **3,** 295–317.
12. Baydoun, E. A.-H. & Fry, S. C. (1985) *Planta* **165,** 269–276.
13. Farmer, E. E., Pearce, G. & Ryan, C. A. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 1539–1542.
14. Casadaban, M. J. & Cohen, S. N. (1980) *J. Mol. Biol.* **138,** 179–207.
15. Hoekema, A., Hirsch, P. R., Hooykaas, P. J. J. & Schilperoort, R. A. (1983) *Nature (London)* **303,** 179–181.
16. Murashige, T. & Skoog, F. (1962) *Physiol. Plant.* **15,** 473–497.
17. An, G., Ebert, P. R., Yi, B.-Y. & Choi, C.-H. (1986) *Mol. Gen. Genet.* **203,** 245–250.
18. An, G. (1986) *Plant Physiol.* **81,** 86–91.
19. An, G. (1987) *Methods Enzymol.* **153,** 292–305.
20. Willmitzer, L. & Wagner, K. G. (1981) *Exp. Cell Res.* **135,** 69–77.
21. Dignam, J. D., Lebovitz, R. M. & Roeder, R. G. (1983) *Nucleic Acids Res.* **11,** 1475–1489.
22. Fried, A. & Crothers, D. M. (1981) *Nucleic Acids Res.* **9,** 6505–6525.
23. Garner, M. M. & Revzin, A. (1981) *Nucleic Acids Res.* **9,** 3047–3059.
24. Winberg, G. & Hammarskjold, M.-L. (1980) *Nucleic Acids Res.* **8,** 253–264.
25. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
26. Ha, S. B. & An, G. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 8017–8021.
27. Lois, R., Dietrich, A., Hahlbrock, K. & Schulz, W. (1989) *EMBO J.* **8,** 1641–1648.
28. Sommer, H. & Seadler, H. (1986) *Mol. Gen. Genet.* **202,** 429–434.
29. Douglas, C., Hoffman, H., Schulz, W. & Hahlbrock, K. (1987) *EMBO J.* **6,** 1189–1195.
30. Feinbaum, R. L. & Ausubel, F. M. (1988) *Mol. Cell Biol.* **8,** 1985–1992.
31. Herrmann, A., Schulz, W. & Hahlbrock, K. (1988) *Mol. Gen. Genet.* **212,** 93–98.
32. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254.