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Systemic accumulation of specific mRNAs in response to wounding in poplar trees

(*Populus*/plant defense/cDNA cloning/gene regulation/chitinase)

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ABSTRACT **cDNAs for several transcripts that accumulate systemically in response to mechanical wounding in a hybrid poplar have been cloned. The corresponding mRNAs become abundant in the unwounded upper leaves of poplar trees whose lower leaves have been damaged. Two of the cDNA clones have been characterized by Northern and Southern blotting, and their nucleotide sequences have been determined. These clones, designated *win6* (wound-inducible) and *win8*, are members of multigene families encoding proteins with a high degree of similarity to chitinases from bean, tobacco, and barley. This initial demonstration of a systemic response to wounding in trees provides an approach to study defense mechanisms in woody plants at the biochemical, physiological, and ecological levels.**

Molecular biology of woody plants is a relatively young discipline, but its application to tree breeding and improvement, forest ecology, and the wood products industry has quickly been recognized (1). Our interest is focused on gene expression and regulation in rapid-growing hybrid poplars developed for the efficient production of biomass for fuel and fiber (2). Poplars have a small genome size (C value = 0.7 pg; ref. 3), are easily propagated vegetatively, and display developmental plasticity in cell culture (4–6). These traits, coupled with susceptibility to *Agrobacterium*-mediated transformation (7, 8) and techniques to regenerate transgenic trees (9, 10), make poplar an attractive model system for forest tree molecular biology.

We have chosen to study changes in gene expression in response to mechanical leaf wounding in poplars for two principal reasons. First, there exists a large body of information on wound-responsive genes in other plant systems. The biochemical defenses of many plants are known to be induced only when the plant is under herbivore or pathogen attack or in response to environmental stress (reviewed in ref. 11). Among these defenses are the production of phytoalexins (12), hydroxyproline-rich glycoproteins (13, 14), "pathogenesis-related" (PR) proteins (15), chitinases (16, 17), glucanases (18), and proteinase inhibitors (19). Mechanical wounding of leaves, by simulating herbivore maceration of leaf tissue, induces a defensive response. Second, a deeper understanding of the wound response in poplars opens the possibility of augmenting the tree's natural defense mechanisms against insect herbivory and pathogen attack. Increasing the levels of phytoalexins, proteinase inhibitors, or hydrolytic enzymes by direct manipulation of the poplar genome may produce more insect- and pathogen-resistant tree varieties. Genes providing protection from pests could be recruited from other organisms, and their expression in transgenic poplars could be regulated by wound-responsive transcription control elements isolated from the tree's own defense genes.

Because we wish to study genes that respond systemically to remote wounding, and not those genes whose transcripts accumulate only in the wounded leaf itself, we have constructed and differentially screened a cDNA library from the unwounded upper leaves of a poplar tree whose lower leaves were mechanically wounded. Two wound-responsive cDNAs were sequenced and used as probes to explore some aspects of the wound response.‡

MATERIALS AND METHODS

Plant Material and Wounding. Hybrid poplar H11-11 (*Populus trichocarpa* × *Populus deltoides; Populus* × *interamericana*) cuttings were rooted in peat/vermiculite, fertilized with Osmocote 14-14-14 (Sierra Chemical, Milpitas, CA), and maintained in a pathogen-free environment at 22°C with a 16-hr day length (a mixture of cool white fluorescent and mercury vapor lamps). The plants were about 75 cm tall, with approximately 30 leaves, when wounded. The wounding was performed on the bottom 20 leaves, and the upper 8 leaves greater than 2 cm in length were collected at the end of the wounding session for RNA isolation. Mechanical wounding of the lower leaves was effected by mashing the leaves between the jaws of a pair of pliers. Two plants of similar age and growth were treated identically, and the harvested leaves were pooled for RNA extraction. Wounding was repeated eight times within a 43-hr period (at times 0, 4, 17, 20, 24, 27, 38, and 40 hr) after which the leaves were excised and immediately dropped into liquid nitrogen, and RNA was extracted from the leaves. Each wounding episode throughout the 43 hr consisted of 15–20 plier "bites" per leaf. Control trees were not wounded. To test for a volatile tree–tree communication signal, wounding of three plants was performed as above in a small (12 m^3) closet, with two control plants placed in separate pots immediately adjacent to the wounded plants.

Nucleic Acid Isolation. Excised leaves were quickly frozen in liquid nitrogen and ground with a mortar and pestle to a fine powder. One milliliter of extraction buffer (100 mM Tris, pH 8.0/20 mM EDTA/0.5 M NaCl/0.5% SDS/0.5% 2-mercaptoethanol) per gram of fresh leaf weight was mixed with one-fifth volume of buffer-equilibrated phenol and warmed to 60°C. The extraction buffer/phenol was then added to the frozen cell powder and agitated under hot running water. One-half volume of chloroform was added, and the mixture was extracted. The aqueous phase was removed, and one-fifth volume of 10 M LiCl was added to

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Abbreviation: PR, pathogenesis related.

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‡The sequences reported in this paper have been deposited in the GenBank data base (accession nos. M25336–M25344).

precipitate the RNA. After 10 min on ice, the RNA was recovered by centrifugation at 10,000 × *g* for 10 min, dissolved in TE (10 mM Tris, pH 7.5/1 mM EDTA), reprecipitated by the addition of one-third volume of 10 M ammonium acetate and two volumes of ethanol, and dissolved in TE at a concentration of 1–5 mg/ml. Poly(A)$^+$ RNA was isolated by oligo(dT)-cellulose affinity chromatography (20). DNA was precipitated from the LiCl supernatant with one volume of isopropanol and purified by banding in CsCl.

cDNA Library Construction and Screening. A cDNA library from the unwounded leaves of wounded trees was synthesized and screened by conventional methods (20). Double-stranded cDNA was cleaved with *Mbo* I and ligated into the *Bam*HI site of M13mp19. Replica filter lifts were taken from each plate: one set was hybridized to a ^{32}P-labeled cDNA probe made by oligo(dT)-primed reverse transcription of total RNA from wounded plants and the other set was hybridized to a probe from nonwounded plants. Clones hybridizing exclusively to cDNA from wounded plants were plaque-purified, and their wound-responsiveness was verified by Northern blot analysis. A full-length cDNA library was constructed in λgt10 by using a cDNA synthesis kit (Pharmacia) and was screened with wound-specific probes from the M13 library.

Southern and Northern Blot Analysis. Blot hybridizations to DNA or RNA immobilized on nylon membranes (MSI, Westborough, MA) were performed according to Church and Gilbert (21).

Nucleotide Sequence Determination. DNA sequences were determined by using the chain termination method (22) with Sequenase (United States Biochemical). M13mp18 templates for the sequencing reactions were generated by the sonication/shotgun cloning method (23). Sequence data were assembled with the DNASTAR software package (DNASTAR, Madison, WI). All nucleotide sequence data has been submitted to GenBank. Accession numbers are given in parentheses following the first mention of each sequence.

Amino Acid Sequence Alignments. DNA sequences were translated by using the GENEPRO program (Riverside Scientific, Seattle, WA) and compared to the sequences in the Protein Identification Resource (1989) Release 19.0 (24) and GenBank (1989) Release 57.0 (25) databases by using the FASTA and TFASTA similarity search programs (26). Sequences were aligned pairwise with these programs, and multiple alignments were performed by eye based on the pairwise alignments.

RESULTS

Isolation of Wound-Specific cDNAs by Differential Screening. Of 4000 recombinant M13 plaques oriented in the proper direction for hybridization to reverse-transcribed probe, 110 displayed wound-specific hybridization on the first round of differential screening. Twenty-five plaques containing probable wound-specific clones were picked for further analysis and subjected to two subsequent rounds of plaque purification and differential screening. Of these, 16 purified plaques (designated clones 1–16) were obtained that hybridized only to probe made from wounded plants. One M13 clone (clone 24) was picked that hybridized equally to probe from wounded and nonwounded trees and was used as a control for equal loading of RNA in Northern blots. Partial DNA sequence information was obtained for 14 of the wound-specific M13 clones to determine if some of the same cDNA fragments were cloned more than once. Clones 2, 3 (accession no. M25339), and 5 are apparently identical to each other and are very similar to clones 11 (M25341) and 14 (M25343). Clones 1 (M25338), 9, and 15 are equivalent to each other. Clones 6 and 7 are identical and are completely contained within the λgt10 clone *win6* (M25336). Clones 4 (M25340), 8 (contained within the λgt10 clone *win8*; M25337), 12 (M25342), and 16 (M25344) did not show nucleotide sequence similarity to any other wound-responsive clones. Sequence information was not obtained for clones 10 and 13. Clones 6 and 8 were chosen for further characterization.

Accumulation of *win6* and *win8* Transcripts in Leaves of Wounded Trees. RNA was extracted from three additional wounded or nonwounded plants to establish the reproducibility of the wound-induced response. Northern blot analysis confirms that clone 8 mRNA shows a large increase in accumulation following wounding, and clone 6 mRNA accumulates to a lesser extent (Fig. 1). Equal loading of RNA in all lanes was verified by ethidium bromide staining of the gel and by hybridization to probe from the constitutively expressed clone 24 (data not shown).

Test for Volatile Tree–Tree Communication Signals. Two studies have suggested that trees are able to sense when neighboring trees have been wounded and to induce their own defenses in response to a presumed volatile signaling agent (27, 28). To test if either clone 6 or clone 8 mRNA accumulation is

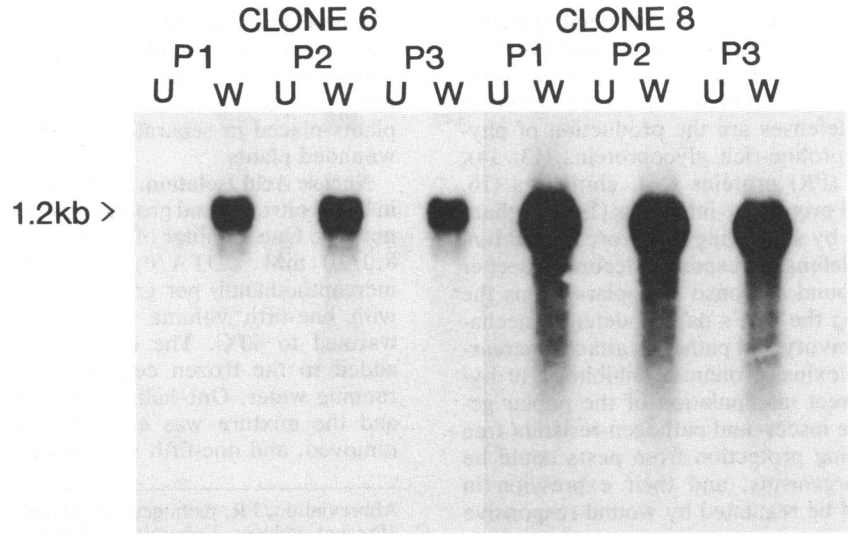

FIG. 1. Accumulation of *win6* and *win8* mRNAs in response to wounding. A Northern blot of total RNA (10 μg per lane) extracted from leaves of unwounded (U) or wounded (W) poplar trees (P1, P2, and P3) and hybridized with probes made from either M13 clone 6 or clone 8 is shown. An RNA ladder (BRL) was used as a molecular size standard.

inducible by proximity to wounded plants, two control plants were placed adjacent to three plants that were severely wounded. The wounded plants displayed a full induction of clone 6 and clone 8 mRNAs, whereas the unwounded plants nearby manifested no accumulation (data not shown).

Win6 and Win8 Are Similar to Chitinases. A cDNA library was constructed in λgt10 and screened with M13 clone 6 and clone 8 probes to isolate full-length cDNAs. A nearly full-length cDNA clone was obtained for clone 8, and a partial cDNA was obtained for clone 6. These λgt10 clones are called *win6* (wound inducible) and *win8*. Each cDNA contains a single large open reading frame. The predicted amino acid sequences were used to query both the Protein Identification Resource and GenBank data bases with FASTA or TFASTA (26). The amino acid sequences of Win6 and Win8 display striking similarity to those from chitinases of bean (29), tobacco (30), and barley (31); additionally, the amino terminus of Win8 shows some similarity to several other proteins: wheat agglutinins I and II (32), rubber tree hevein (33), lectins from rice and great nettle (34), and wound-induced proteins Win1 and Win2 from potato (35) (Fig. 2).

***win* Gene Family Structure.** Southern blots were performed to study the arrangement of the genes that give rise to the *win* transcripts. The results suggest that *win6* and, perhaps, *win8*

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                      10        20        30V       40        50        60
BEAN CHIT  MKKNRMMMMIWSVGVVWM---LLLVG-GSYGEQCGRQAGGALCPGGNCCSQFGWCGSTTD
TOB  CHIT                          SLLLL-SAS-AEQCGSQAGGARCASGLCCSKFGQCGNTND
Win8            EMRFWALTVLSLLLSLLL-GVSSDTAQCGSQAGNATCPNDLCCSSGGYCGLTVA
HEVEIN                                  EQCGRQAGGKLCPNNLCCSQWGWCGSTDE
WHEAT LECT                            ...KRCGSQAGGATCPNNHCCSQYGHCGFGAE
POT Win2                              ...QQCGRQRGGALCGNNLCCSQFGWCGSTPE

                      70        80        90        100       110       120
BEAN CHIT  YCGPG-CQSQC-GGP-SPAPTDLSALISRSTFDQMLKHRNDGACPAKGFYTYDAFIAAAK
TOB  CHIT  YCGPGNCQSQCPGGPTPPGGGDLGSIISSSMFDQMLKHRNDNACQGKGFYSYNAFINAAR
Win8       YCCAG-CVSQC-----------RNCFFTESMFEQMLPNRNNDSCPGKGFYTYDAYFVATE
HEVEIN     YCSPD-HNCQSNCKD
WHEAT LECT YCGAG-CQ...
POT Win2   YCSP...
BARL CHIT                          SVSSIVSRAQFDRMLLHRNDGATQAKGFYTYDAFVAAAA
Win6                                                                   E

                      130       140       150       160       170       180
BEAN CHIT  AYPSFGNTGDTATRKREIAAFLGQTSHETTGGWATAPDGPYAWGYCFVRERNPST-YCSA
TOB  CHIT  SFPGFGTSGDTTARKREIAAFFAQTSHETTGGWATAPDGPYAWGYCWLREQGSPGDYCTP
Win8       FYPGFGMTGDDDTRKRELAAFFAQTSQETSGRSIIGEDAPFTWGYCLVNELNPNSDYCDP
BARL CHIT  AFPGFGRTGSADARKM
Win6       F-PDFGNTGDDLMRKREIAAFLGQTSHETTGGWPDAPCGPYAWGYCYLKEINCQP-YCDP

                      190       200       210       220       230       240
BEAN CHIT  TPQRPCAPGQQYYGRGPIQISWNYNYGQCGRAIGVDLLNKPDLVATDSVISFKSALWFWM
TOB  CHIT  SGQWPCAPGRKYFGRGPIQISHNYNYGPCGRAIGVDLLNNPDLVATDPVISFKSALWFWM
Win8       KTKSSYPCVADYYGRGPLQLRWNYNYGECGNYLGQNLLDEPEKVATDPVLSFEAALWFWM
Win6       SSNYQCVAGKQYCGRGPIQLSWNYNYGLCGDDLKLPLLQEPELVETDPVISFKTAIWFWM

                      250       260       270       280       290       300
BEAN CHIT  TAQSPK-PSSHDVITSRWTPSSADVAARRLPGYGTVTNIINGGLECGR-GQDSRVQDRIG
TOB  CHIT  TPQSPK-PSCHDVIIGRWQPSSADRAANRLPGFGVITNIINGGLECGR-GTDSRVQDRIG
Win8       NPHSTGAPSCHEVITGEWSPSEADILEAGRKPGFGMLTNIITNGGECTKDGK-TRQQNRID
Win6       KPQSPK-PSCHAVITGNWTPSAADLEAGRVPGYGVITNIINGGIECGQGGPNAANEDRIG
BARL CHIT  TAQPPK-PSSHAVIAGQWSPDGADRAAGRVPGFGVITNIIN

                      310       320       330
BEAN CHIT  FFKRYCDLLGVGYGNNLDCYSQTPFGNSLLLSDLVTSQ
TOB  CHIT  FYRRYCSILGVSPGDNLDCGNQRSFGN-GLLVD--TM
Win8       YYLRYCDMLQVDPGDNLYCDNQETFEDNGLLKMVGTM
Win6       FYKKYCDSLGTTYGSNLDCYQQRPFGY-GLSGLKDTM
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FIG. 2. Sequence similarity of Win6 and Win8 to chitinases and other plant proteins. Chitinase from bean (BEAN CHIT) (29) is used as a reference; amino acid sequence identities with bean chitinase are in boldface; sequence identities not shared with bean chitinase are in boldface italics. The signal peptidase cleavage site is indicated (V). Aligned amino acid sequences are tobacco chitinase (TOB CHIT) (30), fragments of barley chitinase (BARL CHIT) (31), rubber tree hevein (33), a partial sequence from wheat isolectin II (WHEAT LECT) (32), and a partial sequence from the wound-responsive gene product Win2 from potato (POT Win2) (35). For hevein, wheat isolectin II, and Win2, only the region of best alignment with chitinases and Win8 is shown. Not shown are alignments with other lectins [wheat isolectin I (32) and rice and great nettle (34)], which are similar to that shown for wheat isolectin II, or alignment of potato Win1, which is similar to that shown for potato Win2.

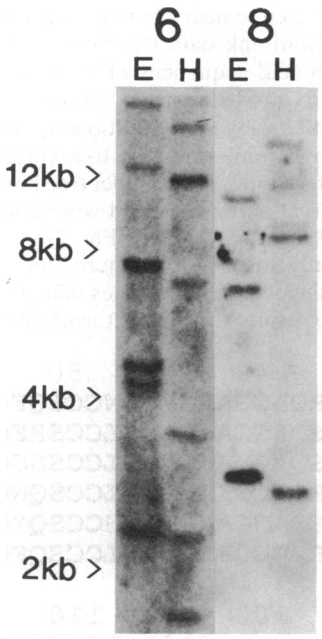

FIG. 3. *win6* and *win8* gene family structure. A Southern blot of DNA (7 μg per lane) extracted from leaves of hybrid poplar H11-11 is shown. The DNA was digested with *Eco*RI (E) or *Hin*dIII (H) and hybridized with probes made from M13 clone 6 (left lanes) or clone 8 (right lanes). A 1-kb ladder was used as a molecular size standard.

are each represented by a family of genes in the poplar genome (Fig. 3). Multiple hybridizing bands are visible with each probe, and the small size of the clone 6 and clone 8 probes makes it unlikely that several *Eco*RI and *Hin*dIII sites would be found within such a short region. These data suggest that sequences hybridizing to the probes are present in multiple copies in the genome; however, the multiple bands conceivably could be due to large intron(s) within the genomic sequences spanned by the *win* cDNA probes. In considering the nature of these *win* gene families, it is necessary to take into account the fact that H11-11 is an interspecific hybrid and is therefore likely to be heterozygous at many loci. Some of the apparent complexity of the *win6* gene family may be due to restriction fragment length polymorphism between *P. trichocarpa* and *P. deltoides*.

DISCUSSION

The results presented here demonstrate the existence of a strong systemic response in poplar plants that is elicited in unwounded upper leaves by the mechanical wounding of lower leaves. Several classes of transcripts accumulate in response to repeated wounding. Systemic response to wounding is a characteristic of another class of defense genes, the proteinase inhibitors of solanaceous plants. In the latter system, the wound response can be elicited by cell wall fragments produced by an endogalacturonidase, which is itself released upon cellular damage (36).

A systemic accumulation of PR proteins is seen in tobacco plants inoculated with tobacco mosaic virus and promotes resistance to subsequent bacterial, fungal, and viral infection (15). Although the function of most of the PR proteins is unknown, a number of PR proteins from potato have recently been identified as chitinases and β-glucanases (37), and four tobacco (16) and four maize (38) PR proteins also were demonstrated to have chitinase activity. The chitinase PR proteins are known to be induced by pathogen attack, fungal elicitor, ethylene, and, locally, by wounding (17, 29).

Two of the mRNAs that accumulate in response to wounding in poplars, *win6* and *win8*, encode proteins that are clearly similar to chitinases from other plants. When aligned with a full-length amino acid sequence of a bean chitinase (Fig. 2), the amino acid sequence derived from the *win8* cDNA extends well into the 27-residue signal peptide of the bean chitinase. Win6 and Win8 chitinases are 53% identical to each other, which is about the same degree of overall similarity that each of these poplar proteins shares with chitinases from bean and tobacco. In several plants, different forms of chitinases are known to accumulate in vacuoles and within cell walls and are encoded by small multigene families (29, 37). This is consistent with the gene arrangement seen in poplars for *win6* and *win8*.

Several lectins (wheat, rice, and nettle) and three proteins of unknown function (rubber tree hevein as well as Win1 and Win2 from potato) show local sequence similarity to Win8. It seems probable that the relatively small region of sequence identity between chitinases and these lectins (and proteins of unknown function) may be attributed to a common carbohydrate-binding domain. The wheat agglutinins specifically bind *N*-acetyl-D-glucosamine, the chitin monomer (39). Relatedness between a wheat lectin and chitinases (40) and the region of sequence similarity between chitinases, hevein, the lectins, and wound-induced gene products from potato (35) have been reported.

Chitinases hydrolyze the β-1,4 linkages of chitin (polymeric *N*-acetyl-D-glucosamine), and plant chitinases often exhibit lysozyme activity as well (41, 42). Since chitinases are known to be potent inhibitors of fungal growth at physiological concentrations, it is thought that chitinase induction serves to protect plants from fungal infection (43, 44).

The mechanism responsible for the accumulation of the *win* mRNAs is not known. One hypothesis is that the increased mRNA levels reflect an increase in the rate of transcription of the corresponding *win* genes. There is ample precedent for this in the induction of plant defensive genes: genes coding for enzymes in the isoflavanoid phytoalexin biosynthetic pathway (45, 46) and for proteinase inhibitors (47) are induced at the transcriptional level. Alternatively, the increased mRNA levels in wounded poplars might be due to a decrease in the turnover rate of the transcripts. This mechanism has not been reported for other defensive genes.

We have tested the ability of unwounded poplars to sense the wounded state of neighboring trees, a communication that has been postulated to occur based on two studies. In one case, leaf palatability to insects has been reported to be reduced on healthy Sitka willows (*Salix sitkensis*) adjacent to defoliated trees (27); in the other, unwounded *Populus* × *euramericana* trees have higher levels of extractable phenolics (presumably defensive) when placed next to wounded trees than when grown in separate quarters (28). The existence of a volatile signal emitted by wounded trees and perceived by neighboring trees has been invoked to explain these data. Our attempts to observe increased accumulation of *win* transcripts in unwounded trees in proximity to wounded trees were unsuccessful. It is unlikely that the wound response is mediated wholly by a volatile agent, since the assayed leaves of the unwounded control plants were no further from the wounded leaves of their neighboring plants than were the assayed (unwounded) leaves of the wounded plants themselves and presumably would be exposed to a similar concentration of any volatile agent.

Further studies on the systemic wound response in poplars that are of interest include the isolation of *win* genomic clones and characterization of their attendant transcriptional control regions in transgenic trees; such studies have recently been done for wound-responsive genes in other plants (48–51). Fusions between *win* transcriptional controls and β-glucuronidase (52) in transgenic trees would allow the study

of wound-response physiology in whole plants under field conditions of insect herbivory and pathogen attack; characterization of this system might lead to tree improvement by increasing the normal wound response or by controlling plant protective genes with *win* transcription signals.

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1. Haissig, B. E., Nelson, N. D. & Kidd, G. H. (1987) *Bio/Technology* **5**, 52–59.
2. Stettler, R. F., Heilman, P. E., Fenn, R. C. & Stanton, B. J. (1988) *Can. J. For. Res.* **18**, 745–753.
3. Dhillon, S. S., Miksche, J. P. & Cecich, R. A. (1984) *Plant Physiol.* Suppl. **75**, 120.
4. Wolter, K. E. (1968) *Nature (London)* **219**, 509–510.
5. Chalupa, V. (1974) *Biol. Plant. (Praha)* **16**, 316–320.
6. Ahuja, M. R. (1983) *Silvae Genet.* **32**, 131–135.
7. DeCleene, M. & DeLey, J. (1976) *Bot. Rev.* **42**, 389–466.
8. Parsons, T. J., Sinkar, V. P., Stettler, R. F., Nester, E. W. & Gordon, M. P. (1986) *Bio/Technology* **4**, 533–537.
9. Fillatti, J. J., Sellmer, J., McCown, B., Haissig, B. & Comai, L. (1987) *Mol. Gen. Genet.* **206**, 192–199.
10. Pythoud, F., Sinkar, V. P., Nester, E. W. & Gordon, M. P. (1987) *Bio/Technology* **5**, 1323–1327.
11. Collinge, D. B. & Slusarenko, A. J. (1987) *Plant Mol. Biol.* **9**, 389–410.
12. Lawton, M. A., Dixon, R. A., Hahlbrock, K. & Lamb, C. J. (1983) *Eur. J. Biochem.* **129**, 593–601.
13. Chen, J. & Varner, J. E. (1985) *EMBO J.* **4**, 2145–2151.
14. Corbin, D. R., Sauer, N. & Lamb, C. J. (1987) *Mol. Cell. Biol.* **7**, 4337–4344.
15. van Loon, L. C. (1985) *Plant Mol. Biol.* **4**, 111–116.
16. Legrand, M., Kauffmann, S., Geoffroy, P. & Fritig, B. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 6750–6754.
17. Hedrick, S. A., Bell, J. N., Boller, T. & Lamb, C. J. (1988) *Plant Physiol.* **86**, 182–186.
18. Kauffmann, S., Legrand, M., Geoffroy, P. & Fritig, B. (1987) *EMBO J.* **6**, 3209–3212.
19. Ryan, C. A. (1983) in *Variable Plants and Herbivores in Natural and Managed Systems*, eds. Denno, R. & McClure, M. (Academic, New York), pp. 43–60.
20. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).
21. Church, G. M. & Gilbert, W. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 1991–1995.
22. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463–5468.
23. Deininger, P. L. (1983) *Anal. Biochem.* **129**, 216–220.
24. George, D. G., Barker, W. C. & Hunt, L. T. (1986) *Nucleic Acids Res.* **14**, 11–15.
25. Bilofsky, H. S., Burks, C., Fickett, J. W., Goad, W. B., Lewitter, F. I., Rindone, W. P., Swindell, C. D. & Tung, C. S. (1986) *Nucleic Acids Res.* **14**, 1–4.
26. Pearson, W. R. & Lipman, D. J. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 2444–2448.
27. Rhoades, D. F. (1983) *ACS Symp. Ser.* **208**, 55–68.
28. Baldwin, I. T. & Schultz, J. C. (1983) *Science* **221**, 277–279.
29. Broglie, K. E., Gaynor, J. J. & Broglie, R. M. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 6820–6824.
30. Shinshi, H., Mohnen, D. & Meins, F. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 89–93.
31. Leah, R., Mikkelsen, J. D., Mundy, J. & Svendsen, I. (1987) *Carlsberg Res. Commun.* **52**, 31–37.
32. Wright, C. S., Gavilanes, F. & Peterson, D. L. (1984) *Biochemistry* **23**, 280–287.
33. Walujuno, K., Scholma, R. A., Beintema, J. J., Mariono, A. & Hahn, A. M. (1976) in *Proceedings of the International Rubber Conference* (Kuala Lumpur), pp. 518–531.
34. Chapot, M.-P., Peumans, W. J. & Strosberg, A. D. (1986) *FEBS Lett.* **195**, 231–234.
35. Stanford, A., Bevan, M. & Northcote, D. (1989) *Mol. Gen. Genet.* **215**, 200–208.
36. Ryan, C. A. (1989) in *Perspectives in Biochemistry*, ed. Neurath, H. (Am. Chem. Soc., Washington, DC), Vol. 1, pp. 112–116.
37. Kombrink, E., Schroder, M. & Hahlbrock, K. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 782–786.
38. Nasser, W., de Tapia, M., Kauffmann, S., Montasser-Kouhsari, S. & Burkard, G. (1988) *Plant Mol. Biol.* **11**, 529–538.
39. Etzler, M. E. (1985) *Annu. Rev. Plant Physiol.* **36**, 209–234.
40. Raikhel, N. W. & Wilkins, T. A. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 6745–6749.
41. Boller, T., Gehri, A., Mauch, F. & Vogeli, U. (1983) *Planta* **157**, 22–31.
42. Metraux, J. P., Burkhart, W., Moyer, M., Dincher, S., Middlesteadt, W., Williams, S., Payne, G., Carnes, M. & Ryals, J. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 896–900.
43. Schlumbaum, A., Mauch, F., Vogeli, U. & Boller, T. (1986) *Nature (London)* **324**, 365–367.
44. Mauch, F., Mauch-Mani, B. & Boller, T. (1988) *Plant Physiol.* **88**, 936–942.
45. Cramer, C. L., Bell, J. N., Ryder, T. B., Bailey, J. A., Schuch, W., Bolwell, G. P., Robbins, M. P., Dixon, R. A. & Lamb, C. J. (1985) *EMBO J.* **4**, 285–289.
46. Cramer, C. L., Ryder, T. B., Bell, J. N. & Lamb, C. J. (1985) *Science* **227**, 1240–1243.
47. Graham, J. S., Hall, G., Pearce, G. & Ryan, C. A. (1986) *Planta* **169**, 399–405.
48. Thornburg, R. W., An, G., Cleveland, T. E., Johnson, R. & Ryan, C. A. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 744–748.
49. An, G., Mitra, A., Choi, H. K., Costa, M. A., An, K., Thornburg, R. W. & Ryan, C. A. (1989) *Plant Cell* **1**, 115–122.
50. Logemann, J., Lipphardt, S., Lorz, H., Hauser, I., Willmitzer, L. & Schell, J. (1989) *Plant Cell* **1**, 151–158.
51. Teeri, T. H., Lehvaslaiho, H., Franck, M., Uotila, J., Heino, P., Palva, E. T., Van Montagu, M. & Herrera-Estrella, L. (1989) *EMBO J.* **8**, 343–350.
52. Jefferson, R. A. (1987) *Plant Mol. Biol. Rep.* **5**, 387–405.