The EMBO Journal vol.6 no.2 pp.303–306, 1987

Wound-induced expression of a potato proteinase inhibitor II gene in transgenic tobacco plants

Jose J.Sanchez-Serrano, Michael Keil, Aileen O'Connor, Jeff Schell and Lothar Willmitzer[1]

Max-Planck-Institut für Züchtungsforschung, 5000 Köln 30, FRG

[1]Present address: Institut für Genbiologische Forschung Berlin, 1000 Berlin 33, FRG

Communicated by J.Schell

A potato proteinase inhibitor II gene was transferred into tobacco plants using *Agrobacterium*/Ti-plasmid-mediated gene transfer techniques. Whereas no or little expression of the proteinase inhibitor II gene could be detected in non-wounded leaves, high levels of proteinase inhibitor II mRNA were detected in leaves of several transgenic tobacco plants after mechanical wounding as well as after treatment of detached leaves with oligosaccharides. Wounding of a leaf also led to a systemic induction in non-wounded leaves as well as non-wounded stem, and roots. RNA-protection experiments showed that the transcription of the proteinase inhibitor gene in transgenic tobacco plants was initiated at the same nucleotide as that of the original gene. These observations demonstrate that although no proteinase inhibitor II homologous gene can be detected in tobacco, this plant nevertheless has the capacity to regulate the expression of the potato gene in the same complex manner as in the case of potato.

Key words: tobacco/potato proteinase inhibitor II /gene expression/induction/wounding

Introduction

The wound response of plants has been extensively analyzed at the physiological level over the last few years. Wounding by either mechanical means or by a pathogen, results in a variety of changes in the physiological state of the tissue. The production of cell-wall components such as lignin and suberin is increased in order to try to close the wounded surface as rapidly as possible (cf. Sequeira, 1983). In the case of a simultaneous pathogen attack, low mol. wt substances are produced at least some of which are inhibitory to pathogen growth (Misaghi, 1982).

The defense reaction of the plant against wounding is either restricted to a defined region in the near vicinity of the original wound (local reaction) such as in the case of the production of phytoalexins; or may also occur in tissues far from the original wound site (systemic reaction). In the case of a systemic reaction a diffusible factor has to be postulated which signals the event (wounding) to more distant tissues in the plant. One of the best studied cases of a systemic reaction induced in higher plants by wounding is the accumulation of proteinase inhibitors in leaves of tomato and potato after mechanical wounding or wounding by chewing insects (Green and Ryan, 1972; Ryan, 1977).

In non-wounded plants proteinaceous proteinase inhibitors are most commonly found in storage organs such as seeds and tubers. Whereas there can be no doubt about their gross physiological function, i.e. prevention of unwanted proteolysis, their detailed physiological function is poorly understood. One of the most interesting ideas is that they might play a role in the defence strategy of plants against insect or microbial attack by inhibiting the respective pathogen proteinase. This assumption is based on the three following observations. (i) The inhibitory activity of plant proteinaceous proteinase inhibitors is directed against a wide range of proteases of microbial or insect origin, but rarely against proteases of plant origin (Richardson, 1977). (ii) Plant proteinase inhibitors accumulate preferentially in organs important for either vegetative or sexual propagation such as seeds or tubers. (iii) The accumulation of at least some proteinase inhibitors can be induced in non-storage organs by wounding, thereby mimicking the action of a chewing insect.

As already outlined above, the accumulation of the proteinase inhibitors is not restricted to the actual wounding site but also occurs in more distal tissues. Extensive biochemical work has led to the isolation and characterization of a 'proteinase inhibitor-induced factor' called PIIF probably responsible for the systemic reaction. It is composed of several oligosaccharides of different chain length and sugar composition probably produced by the action of endopolygalacturonases on the plant cell wall (Bishop *et al.*, 1981).

This factor induces a number of proteinaceous protease inhibitors in potato as well as in tomato plants, the best studied cases being the proteinase inhibitors I and II from potato and tomato which display a chymotrypsin- and trypsin/chymotrypsin-inhibiting activity respectively. We have recently isolated cDNA's as well as genomic clones encoding proteinase inhibitor II of potato (Sanchez-Serrano *et al.*, 1986; Keil *et al.*, 1986). Using these clones we showed that in potato the expression of the proteinase inhibitor II gene family is under developmental as well as environmental control, i.e. being tuber specific in a non-wounded potato plant (Sanchez-Serrano *et al.*, 1986 Rosahl *et al.*, 1986) and induced by mechanical wounding in leaves (Sanchez-Serrano *et al.*, 1986).

Tobacco plants do not have sequences homologous to the proteinase inhibitor II of potato and could therefore be used to test whether, and under which conditions, the potato gene can be expressed in transgenic plants. The results presented here indicate that tobacco plants, although devoid of a proteinase inhibitor II gene, are nevertheless capable of expressing the potato gene specifically after wounding.

Results

The structure of the proteinase inhibitor II gene transferred into tobacco is shown in the lower part of Figure 1. Tobacco was chosen as the host plant as it does not contain any detectable homology to the proteinase inhibitor II gene family (cf. Figure 1, upper part, lane 1). The *Eco*RI genomic fragment transferred contained 3 kb of 5′-upstream and 1.45 kb of 3′-downstream sequences in addition to the coding region and was cloned into the intermediate vector pMPK110 (Eckes *et al.*, 1986) and subsequently mobilized by triparental mating (Van Haute *et al.*, 1983) into the *Agrobacterium* host strain C58C1 (pGV3850 Kan^R) (Jones *et al.*, 1985). The structure of the cointegrate formed with

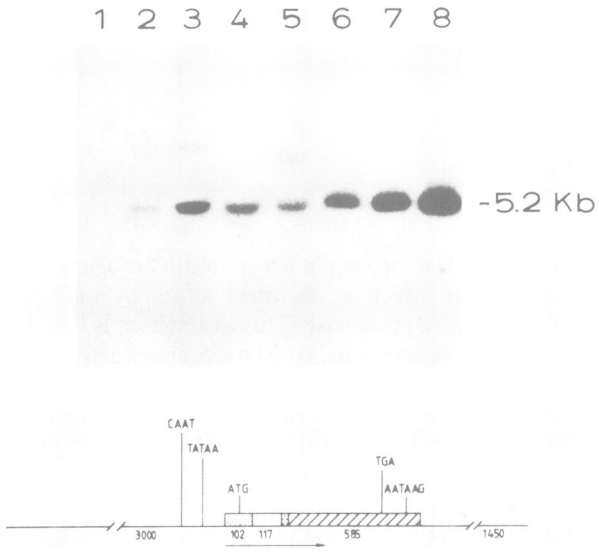

Fig. 1. Upper part: Southern blot analysis of an untransformed and four independently transformed tobacco plants. 10 μg total DNA extracted from one untransformed (**lane 1**) and four independently transformed tobacco plants (**lanes 2–5**), were digested by *Eco*RI and transferred to nitrocellulose filters after gel electrophoretic separation. The autoradiogram shows the hybridization pattern obtained using a nick-translated probe containing a potato proteinase inhibitor II cDNA (cDNA 1) (Sanchez-Serrano *et al.*, 1986). **Lanes 6–8** show the results of a reconstruction experiment where plasmid DNA equivalent to 5 (**lane 6**), 10 (**lane 7**) or 25 (**lane 8**) copies/haploid genome was offered for hybridization. **Lower part**: schematic structure of the proteinase inhibitor II gene. Exons are indicated by filled boxes, the intron is shown as an open box. The signal peptide (cf. Sanchez-Serrano *et al.*, 1986) is indicated by a dotted box. The lengths of the exons and the intron are given in nucleotides.

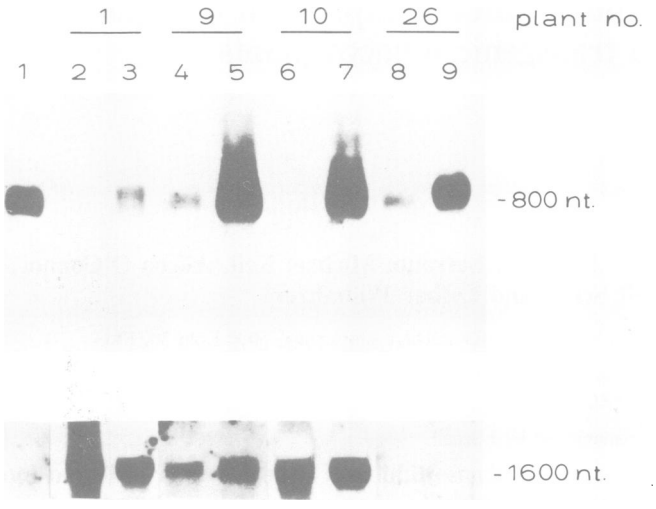

Fig. 2. Influence of wounding on the expression of the proteinase inhibitor II gene in leaves of transgenic tobacco plants. Tobacco plants (size: 80 cm) were wounded on one leaf by two consecutively applied dialysis clamps the second clamp being applied 20 h after the first. RNA was isolated from the wounded leaf 24 h after the first clamp was attached. For the non-wounded control a leaf was harvested from the same plant at the time of applying the first wound. Wounded and non-wounded leaves were always of about the same size and age. Total RNA (50 μg each) isolated from wounded leaves of potato (**lane 1**), non-wounded leaves (**lanes 2, 4, 6** and **8**) and wounded leaves (**lanes 3, 5, 7** and **9**) of the transgenic tobacco plants nos. 1, 9, 10 and 26 was subjected to gel electrophoretic separation using formaldehyde gels and hybridized with a potato proteinase inhibitor II cDNA clone (cDNA 1) (Sanchez-Serrano *et al.*, 1986). The lower part shows the result obtained after hybridizing the same RNAs with a nick-translated probe of the nopaline synthese gene. In plant no. 26 expression of the nopaline synthase gene is very weak.

the Ti plasmid was checked by Southern blot hybridization (data not shown). Subsequently leaf discs of *Nicotiana tabacum* cv. W 38 were infected with the *Agrobacterium* strain containing the proteinase inhibitor II gene. Transformed shoots were selected on 100 μg/ml kanamycin and regenerated plantlets were subsequently transferred to the greenhouse for further analysis. The upper part of Figure 1 (lanes 2–5) shows the Southern blot analysis of four independently transformed tobacco plants which were also used in the expression experiments described below. In the *Eco*RI digest all plants display a 5.2-kb long fragment hybridizing to a cDNA (Sanchez-Serrano *et al.*, 1986) of the proteinase inhibitor II gene. This size would be expected for a non-rearranged fragment containing the proteinase inhibitor II gene. Reconstruction experiments (lanes 6–8) indicate that the copy number of the transferred gene varies between one and three copies/haploid genome. No hybridization was observed with DNA isolated from non-transformed tobacco (lane 1). Ten independently transformed plants containing at least one copy of the non-rearranged proteinase inhibitor II gene were analyzed for expression of this gene with and without wounding of leaves. Northern gel type hybridizations did not reveal any or only very low levels of RNA homologous to the inhibitor II gene in non-wounded leaves (cf. Figure 2, lanes 2, 4, 6, 8 and data not shown). However, when the RNA was isolated from leaves which had been exposed to two consecutive woundings, four out of the ten plants analyzed showed a prominent RNA in Northern blot experiments (Figure 2, lanes 3, 5, 7, 9). This RNA co-migrated with the proteinase inhibitor II mRNA from wounded potato leaves (Figure 2, lane 1). In addition the expression of the transferred proteinase inhibitor can be as high in wounded leaves of transgenic tobacco plants as in wounded leaves of potato (cf. lanes 3, 5, 7 and 9 to lane 1 in Figure 2). The same RNAs were subsequently probed for the expression of the nopaline synthase gene which is a closely linked marker gene introduced by the T-DNA vector and separated by 6 kb from the proteinase inhibitor II gene. The results in the lower part of Figure 2 (lanes 2–9) show that the expression of the nopaline synthase gene remains largely invariant or shows a much lower influence of the wounding of the tobacco leaves on its expression. The specific induction of the proteinase inhibitor II gene by wounding strongly suggests that the wound inducibility is controlled by *cis*-acting regulatory sequences within the transferred gene. RNA protection experiments were performed in order to analyze whether the transcription initiation site used in the transgenic tobacco plants is the same as the one used in wounded potato leaves and tubers. To this end, radioactively labelled RNA complementary to the non-coding strand of the first exon as well as to ~1.5 kb of the 5′-upstream sequences of the proteinase inhibitor II gene, was synthesized using the SP6 system (Melton *et al.*, 1984). This RNA was subsequently hybridized to total RNA isolated from either tubers or wounded leaves of potato as well as from wounded and non-wounded leaves of a transgenic tobacco. The size of the RNAse-resistant hybrids was subsequently determined on sequencing gels. The results, shown in Figure 3, demonstrate that transcription starts at the same nucleotide in wounded leaves of transgenic tobacco plants as well as in tubers and in wounded leaves of potato (cf. Figure 3, lanes 2–4). Therefore not only the general control of the expression but also the transcription initiation as well as the processing of the RNA precursor is maintained in leaves of transgenic tobacco plants. Again no protected RNA is seen in the case of the non-wounded transgenic tobacco leaf. This is in agreement with the data shown in Figure 2.

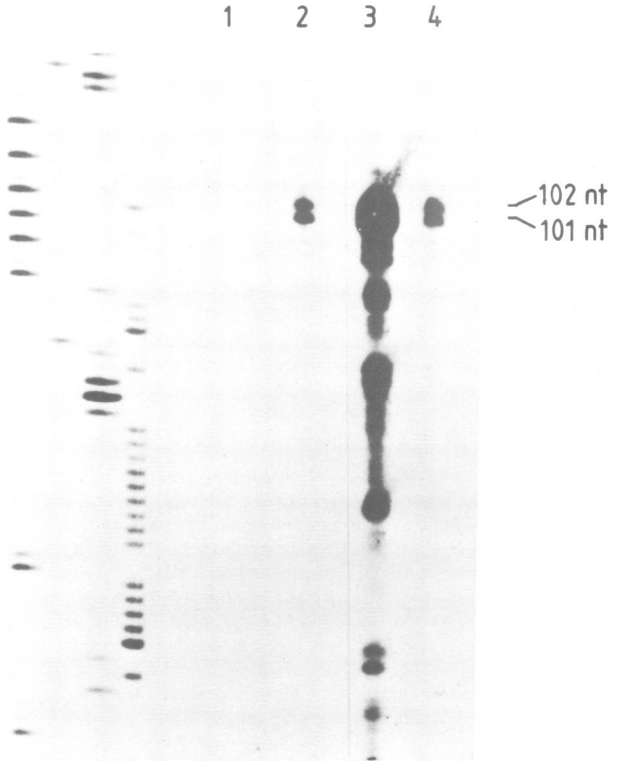

Fig. 3. Mapping of the transcription initiation site of the proteinase inhibitor II gene in transgenic tobacco plants. Total RNA (50 μg) from non-wounded (**lane 1**) and wounded (**lane 2**) leaves of tobacco (plant no. 10), potato tubers (**lane 3**) and wounded potato leaves (**lane 4**) were hybridized against a radioactively labelled RNA complementary to the non-coding strand of the first exon and 1.5 kb of the upstream region of the proteinase inhibitor II gene (Keil *et al.*, 1986). RNA hybrids protected from RNase digestion were separated on a 6% polyacrylamide sequencing gel. The left part of the figure shows a sequencing gel used for size determination. The size of the longest fragments protected is given in nucleotides.

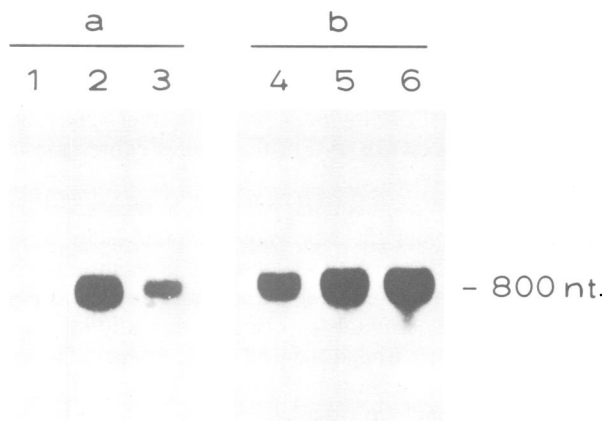

Fig. 4. (**a**) Systemic induction of the proteinase inhibitor II gene by wounding. Tobacco plant no. 10 was wounded on the third and fifth leaf as described in Figure 2 leaving the fourth leaf unwounded. RNA was isolated from the third and fifth leaf (**lane 2**) and the fourth leaf (**lane 3**) 20 h after the first wounding as well as from another leaf shortly before wounding (**lane 1**) and analyzed for expression of the proteinase inhibitor II gene by Northern gels as described in Figure 2; 50 μg of total RNA were applied in each lane. (**b**) Induction of proteinase inhibitor II gene expression by different oligosaccharides. Single leaves derived from plant no. 10 were detached by cutting and exposed to different oligosaccides by supplying a solution of 50 mM sodium phosphate (pH 7.0) containing either 0.2% polygalacturonic acid or 0.2% chitosan or neither for 4 h through the cut petiole. Leaves were incubated subsequently for an additional 12 h in 50 mM sodium phosphate without polysaccharide in continuous light before harvesting. Subsequently total RNA was isolated and analyzed by Northern gels as described in Figure 2. **Lane 4**: RNA (50 μg) extracted from a phosphate-incubated leaf. **Lane 5**: RNA (50 μg) extracted from a chitosan-incubated leaf. **Lane 6**: RNA (50 μg) extracted from a polygalacturonic acid-incubated leaf.

Table I. Homology in the 3'-untranslated regions of an extensin gene (Chen and Varner, 1985) and the proteinase inhibitor II gene (Keil *et al.*, 1986)

30	GGCCAACTTAAGAAG ... ATGAAGTAAAATAATG	60	Extensin
	********** * ***** ***** ***		
26	GGCCAACTTAATTAATGTATGAAATAAAAGGATG	59	Proteinase inhibitor II

The conserved nucleotides are indicated by an asterisk, the numbering refers to the nucleotide position after the respective stop codon.

The factor responsible for the induction of the proteinase inhibitor II gene in tomato has been isolated and shown to be composed of several oligosaccharide fractions probably derived from the plant cell wall (Ryan *et al.*, 1985). The systemic induction of the proteinase inhibitor I (Green and Ryan, 1972) and II (Sanchez-Serrano *et al.* 1986) gene observed after wounding might be mediated by this factor (Ryan *et al.*, 1985). The authentic factor as well as certain substitutes such as chitosan or polygalacturonic acid can also be used to increase the expression of the proteinase inhibitor II gene in detached leaves of tomato (Ryan *et al.*, 1985) and potato (Sanchez-Serrano *et al.*, 1986). The data shown in Figure 4 demonstrate that the proteinase inhibitor II gene transferred into tobacco is systemically induced in exactly the same way as it is in potato, i.e. systemic induction in non-wounded leaves (lane 3) as well as the upper part of the stem and in the root (data not shown). In addition its expression in detached leaves can be increased over and above the level achieved after detachment of the leaf (lane 4), by additional treatment with either chitosan (lane 5) or polygalacturonic acid (lane 6).

In non-wounded potato plants the expression of the proteinase inhibitor II is limited to the tubers. No signal is detectable in leaves, stem, roots or seeds (Rosahl *et al.*, 1986; Sanchez-Serrano *et al.*, 1986) of a non-wounded plant. We were therefore also interested in analyzing whether the proteinase inhibitor II gene would be active in non-wounded organs of the transgenic tobacco plant. RNA isolated from flowers, seeds, stem, and roots of non-wounded transgenic tobacco plants (plants no. 10 and 26) did not show any hybridization to the proteinase inhibitor II gene (data not shown). Taken together the result as well as those shown in Figure 4, demonstrate that the transferred gene is regulated in the same way as it is in potato, being inactive in non-wounded organs of the transgenic plants and being systemically induced in leaves, stem and root segments after wounding.

Discussion

A proteinase inhibitor II gene of potato has been transferred to tobacco cells using *Agrobacterium*/Ti-plasmid-mediated gene transfer techniques. Intact tobacco plants have been regenerated and shown by Southern analysis to contain between one and five copies of the non-rearranged gene. No homology to the proteinase inhibitor gene was found in non-transformed tobacco plants. Whereas no or very little expression of the proteinase inhibitor II gene could be detected in non-wounded leaves, high levels of proteinase inhibitor II mRNA were detected in four out of the ten transgenic tobacco plants tested after a mechanical wounding of the leaves. The expression was not limited to the actual wounding site but was also found in lower non-wounded leaves as well

as part of the stem and roots. In addition an increase in the level of the proteinase inhibitor II mRNA was observed after treatment of detached leaves with solutions containing different oligosaccharides. These data therefore demonstrate that the expression of the proteinase inhibitor II gene in transgenic tobacco plants follows the exact pattern of this gene's expression in its natural host, i.e. potato.

It is reasonable to postulate the presence in tobacco of trans-acting factors controlling the expression of wound-induced genes. Since tobacco is devoid of an endogenous proteinase inhibitor II gene, the induction of this gene in the transgenic plants is supposed to be modulated by tobacco factors normally involved in the expression of other wound-induced tobacco genes. These factors then have to be well conserved among different plant species.

Since it is reasonable to postulate that they interact with *cis*-regulatory sequences located within the gene or in its near vicinity, a sequence comparison was carried out between the proteinase inhibitor II gene and other wound-inducible genes. Homology was observed between the 3′-terminal part of the proteinase inhibitor II gene and a genomic clone from carrot encoding extensin (Chen and Varner, 1985). The longest stretch displaying homology is located in the 3′-untranslated region of the genes ~30 nucleotides after the stop codons. Within a segment of 34 nucleotides 25 nucleotides are conserved with a gap of three nucleotides (cf. Table I). Among these 34 nucleotides the first 11 nucleotides are completely conserved. As extensin is thought to be a ubiquitous protein, one might speculate that the *trans*-acting factors involved in the wound-induced activation of the proteinase inhibitor II gene in tobacco might normally be used for the expression of the tobacco's extensin genes and probably other wound-induced genes. It remains to be shown that the physical homology between the extensin and the proteinase inhibitor II gene is actually meaningful in functional terms.

The identification of *cis*-acting regulatory sequences important for wound-induction might lead to new strategies for obtaining pathogen-resistant plants using gene technology methods by constructing artificial resistance genes whose expression would be limited to instances of actual pathogen attack.

Furthermore it remains to be seen whether the introduction of the proteinase inhibitor II gene into plants devoid of this gene would already be beneficial to the plant in terms of resistance against pathogen attack.

Materials and methods

Recombinant DNA techniques

Standard procedures were used for recombinant DNA work (Maniatis *et al.*, 1982).

Isolation and analysis of nucleic acids

Isolation of RNA from higher plants was performed according to Logemann *et al.* (1986); isolation of DNA from higher plants as well as agrobacteria was carried out according to Murray and Thompson (1980); analysis of the nucleic acids by blot hybridizations was performed according to Eckes *et al.* (1986).

Transfer of the potato proteinase inhibitor gene into tobacco plants

The proteinase inhibitor II gene characterized previously (Keil *et al.*, 1986) together with 3 kb of 5′-upstream and 1.45 kb of 3′-downstream sequences was transferred into tobacco plants by first cloning it into the *Eco*RI site of the intermediate vector pMPK 110 (Eckes *et al.*, 1986) and mobilizing the plasmid obtained into the disarmed *Agrobacterium* strain C58C1 (pGV3850 kan^R) (Jones *et al.*, 1985) by a triparental mating using GJ23 as the mobilizing strain (van Haute *et al.*, 1983). Cointegrates obtained were selected in the presence of streptomycin (300 μg/ml), spectinomycin (100 μg/ml), erythromycin (50 μg/ml) and chloramphenicol (25 μg/ml). Agrobacteria containing the proteinase inhibitor II gene were subsequently used for leaf disc infection of *Nicotiana tabacum* cv. Samsun using established procedures (Horsch *et al.*, 1985). Shoots arising on 100 μg/ml of kanamycin were rooted on hormone-free medium containing 100 μg/ml of kanamycin and subsequently tested for nopaline synthase activity (Otten and Schilperoort, 1978). Only those plants which were both kanamycin resistant and nopaline positive were transferred into the greenhouse and cultivated further. All manipulations were performed according to the Richtlinien für den Umgang mit neukombinierten Nukleinsäurren of the Bundesministerium für Forschung und Technologie.

Wounding experiments

Tobacco plants (size: 80 cm) were wounded on one leaf by two consecutively applied dialysis clamps, the second clamp being applied 20 h after the first according to the procedure described by Green and Ryan (1972). Leaves were harvested 24 h after the first wound was made and total RNA was isolated as described.

Oligosaccharide treatment

Single leaves were detected from the plant by cutting and exposed to different oligosaccharides by supplying a solution of 50 mM sodium phosphate (pH 7.0) containing either 0.2% polygalacturonic acid or 0.2% chitosan for 4 h through the cut petiole. Leaves were subsequently incubated for an additional 12 h in 50 mM sodium phosphate without oligosaccharide in continuous light before harvesting, and total RNA was then isolated.

RNA protection experiments

Radioactively labelled RNA homologous to the non-coding strand of the first exon and 1.5 kb of the upstream region of the proteinase inhibitor II gene (Keil *et al.*, 1986) was synthesized using appropriate SP6 vectors and SP6 RNA polymerase (Melton *et al.*, 1984). Hybridization to total RNA (50 μg) from non-wounded and wounded leaves of transgenic tobacco plants as well as wounded leaves and tubers of potato was carried out for 16 h at 50°C in a buffer containing 80% formamide, 40 mM Pipes, 0.4 M NaCl, 1 mM EDTA, pH 6.7. After hybridization non-protected RNA was digested in a buffer containing 10 mM Tris, pH 7.5, 5 mM EDTA, 300 mM NaCl, 20 μg/ml RNase A and 1 μg/ml RNase T1 for 30 min at 30°C. RNA hybrids protected from digestion were separated on a 6% polyacrylamide sequencing gel.

Acknowledgements

We would like to thank Astrid Blau for help with the plant transformation work, Hugo Peña-Cortés for the root RNA isolation, Mr and Mrs Czerny and Ingrid Reinsch for the plant work, Jutta Freitag for editing the manuscript and Maret Kalda for the photographic work. J.J.S.-S. is a recipient of a fellowship from Fundación Juan March (Spain). This work was supported by a grant from the Bundesministerium für Forschung und Technologie (BCT 0365/2 'Molekulare Gen- und Zelltechnologie'.

References

Bishop,P.D., Pearce,G., Bryant,J.E. and Ryan,C.A. (1981) *J. Biol. Chem.*, **259**, 13172–13177.

Chen,J. and Varner,J. (1985) *Embo J.*, **4**, 2145–2151.

Eckes,P., Rosahl,S., Schell,J. and Willmitzer,L. (1986) *Mol. Gen. Genet.*, **205**, 14–22.

Green,T.R. and Ryan,C.A. (1972) *Science*, **175**, 776–777.

Horsch,R.B., Fry,F.E., Hoffmann,N.L., Eichholtz,D., Rogers,S.G. and Fraley, R.T. (1985) *Science*, **227**, 1229–1231.

Jones,J.D., Dunsmuir,P. and Bedbrook,J. (1985) *EMBO J.*, **4**, 2411–2418.

Keil,M., Sanchez-Serrano,J., Schell,J. and Willmitzer,L. (1986) *Nucleic Acids Res.*, **14**, 5641–5650.

Logemann,J., Schell,J. and Willmitzer,L. (1986) *Anal. Biochem.*, in press.

Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Melton,D.A., Krieg,P.A., Rebagliati,M.R., Maniatis,T., Zinn,K. and Green, M.R. (1984) *Nucleic Acids Res.*, **12**, 7035–7056.

Misaghi,I.J. (1982) *Physiology and Biochemistry of Plant–Pathogen Interactions*. Plenum Press, New York.

Murray,M.G. and Thompson,W. (1980) *Nucleic Acids Res.*, **8**, 4321–4325.

Otten,L. and Schilperoort,A.R. (1978) *Biochim. Biophys. Acta*, **527**, 497–500.

Richardson,M. (1977) *Phytochemistry*, **16**, 159–169.

Rosahl,S., Eckes,P., Schell,J. and Willmitzer,L. (1985) *Mol. Gen. Genet.*, **202**, 368–373.

Ryan,C.A. (1977) *Annu. Rev. Plant Physiol.*, **24**, 173–196.

Ryan,C.A., Bishop,P.D., Walker-Simmons,M., Brown,W.E. and Graham,J.S. (1985) In Key,J. and Kosuge,T. (eds), *UCLA Symposia on Molecular and Cellular Biology, New Series*, **22**, 319–334.

Sanchez-Serrano,J., Schmidt,R., Schell,J. and Willmitzer,L. (1986) *Mol. Gen. Genet.*, **203**, 15–20.

Sequeira,L. (1983) *Annu. Rev. Microbiol.*, **37**, 51–97.

Van Haute,E., Joos,H., Maes,M., Warren,G., Van Montagu,M. and Schell,J. (1983) *EMBO J.*, **2**, 411–418.

Received on 17 November 1986