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5′ Upstream Sequences from the *wun1* Gene Are Responsible for Gene Activation by Wounding in Transgenic Plants

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A 1.2-kilobase pair fragment of the 5′ upstream region of a potato wound-inducible gene (*wun1*) was fused to different marker genes (*wun1*-CAT, *wun1*-NPTII). Stable integration of a *wun1*-CAT chimeric gene into the tobacco genome led to a high wound-inducible chloramphenicol acetyltransferase activity in leaves. Transient expression experiments in potato protoplasts showed that *wun1* carries a strong promoter sequence similar in strength to the ^{35}S promoter. The same intensity of expression was also observed using *wun1* constructs in transient experiments with rice protoplasts. *wun1* mRNA was shown to accumulate to high levels in potato leaves collapsing as a result of infection with the phytopathogen *Phytophthora infestans*. The *wun1* product might, therefore, play a role in a general physiological reaction to stress correlated with cell death.

INTRODUCTION

Wounding leads to drastic changes in the plant's physiology and this is in part a consequence of changes in the expression pattern of several genes. Various wound-induced mRNAs and related proteins have been identified in different plant species, e.g. phenylalanine ammonium-lyase and 4-coumarate:CoA ligase (Chapell and Hahlbrock, 1984; Edwards et al., 1985; Fritzemeier et al., 1987), proteinase inhibitors (Graham et al., 1985; Sanchez-Serrano et al., 1986), thionin (Bohlmann et al., 1988), pathenogenesis-related proteins (Van Loon, 1985; Kauffmann et al., 1987; Legrand et al., 1987; Kombrink et al., 1988), hydroxyproline-rich glycoproteins (Chen and Varner, 1985), as well as chalcone synthase (Ryder et al., 1987) and stilbene synthase (Vornam et al., 1988). Up to now little has been known about the corresponding genes that are responsible for wound-inducible expression.

A carrot extensin genomic clone was characterized and shown to be responsible for the synthesis of two different mRNAs initiated from different transcriptional start points (Chen and Varner, 1985).

Although the PR proteins that are induced in tobacco leaves by tobacco mosaic virus infection have been described in some detail, only in the case of the PR1a gene have the genomic sequence and putative promoter region been analyzed (Cornelissen et al., 1987). However, neither the extensin nor the PR1a gene has been analyzed functionally in transgenic plants.

An example of a functional analysis of a wound-inducible gene is given by the proteinase inhibitor II genes from potato and tomato. It was demonstrated that an intact proteinase inhibitor gene from potato was active after wound induction in transgenic tobacco plants (Sanchez-Serrano et al., 1987). However, the analysis of *cis* elements responsible for wound induction is complicated. The transcriptional fusion of a 5′ upstream region of a proteinase inhibitor II gene with chloramphenicol acetyltransferase (CAT) and with the 3′ end of the T-DNA-derived gene 6 b did not result in a chimeric gene capable of expression in transgenic tobacco plants (Thornburg et al., 1987). Replacing the 3′ sequence of gene 6 b by the proteinase inhibitor 3′ end resulted in a weak CAT activity and a slight wound inducibility (Thornburg et al., 1987).

We present here the functional characterization of the 5′ regulatory region of the wound- and pathogen-inducible gene *wun1* isolated from potato (Logemann et al., 1988). *wun1* mRNA starts accumulating in potato tubers 30 min after wounding, and maximal accumulation is observed between 4 and 24 hr. Wounding of potato leaves, stems, and roots also increases the amount of *wun1* mRNA in these tissues (J. Logemann and J. Schell, manuscript in preparation). *wun1* codes for a small protein of about 18 kD and its expression is regulated on the transcriptional level (Logemann et al., 1988).

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The isolation and functional characterization of the corresponding gene and of transcriptional fusions of its 5′ sequences to different marker genes in transgenic tobacco plants are described here. Transient expression experiments indicate that these 5′ upstream sequences are maintaining their transcription-activating function in different host plants, including a monocot such as rice.

RESULTS

Construction of *wun1*-CAT and *wun1*-NPTII Chimeric Genes

The isolation and characterization of a genomic *wun1* clone from a haploid potato line AM80/5793 is described elsewhere (J. Logemann and J. Schell, manuscript in preparation). The genomic *wun1* clone consists of a 1022-bp upstream region, a 217-bp untranslated 5′ region, a 318-bp coding region, and a 3′ end of 259 bp (Figure 1). To identify *cis*-active regulatory elements, transcriptional fusions to two marker genes (*wun1*-CAT, *wun1*-NPTII) were constructed (Figure 1) and tested in transgenic plants and transiently in protoplasts of different origin.

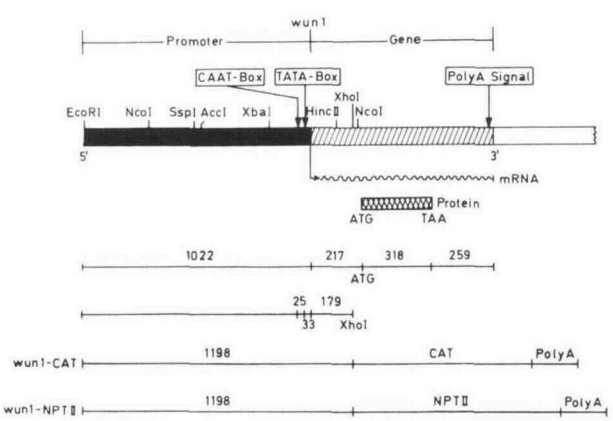

Figure 1. Organization of *wun1* and Description of *wun1*-CAT and *wun1*-NPTII.

Within a 4-kb genomic EcoRI fragment derived from a haploid potato line, 1022 bp of *wun1* 5′ regulatory region (containing CAAT box and TATA box), a 217-bp 5′ untranslated region, a 318-bp coding region, and a 259-bp 3′ untranslated region (containing poly(A) signal) are present. Using the unique XhoI site, the *wun1* 5′ regulatory region (1022 bp) and 176 bp of the 217-bp large 5′ untranslated region were performed for transcriptional fusion to a CAT gene (*wun1*-CAT) or a NPTII gene (*wun1*-NPTII). In the case of *wun1*-CAT, the poly(A) signal of the cauliflower mosaic virus-derived 35S gene was used as a 3′ end. The 3′ end of *wun1*-NPTII is derived from the polyadenylation signal from the octopine synthase gene.

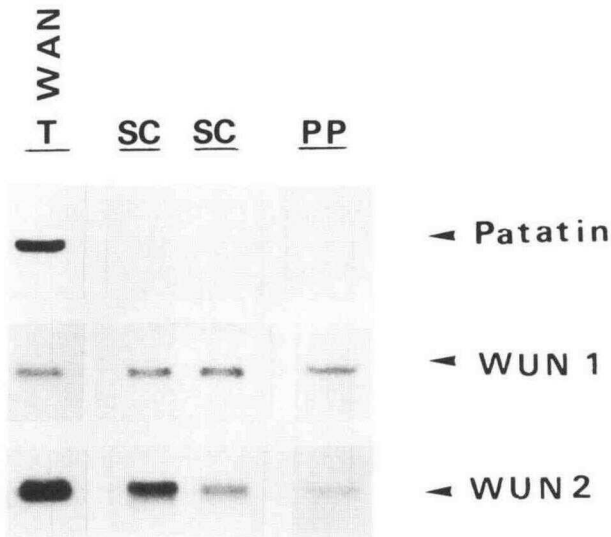

Figure 2. RNA Gel Blot Analysis of Potato Suspension Cultures and Derived Protoplasts.

Fifty μg of total RNA from two identical potato suspension cultures (SC) as well as its protoplasts (PP) were isolated. After gel electrophoretic separation on a 1.2% agarose gel, the RNA was blotted onto a nitrocellulose filter and hybridized against various radioactively labeled cDNA probes (patatin, *wun1*, *wun2*). As a control, 50 μg of RNA from wounded potato tubers grown under anaerobic conditions (TWAN) were used, from which positive gene expression of all three clones was known already (Logemann et al., 1988).

In *wun1*-CAT, 1022 bp of the *wun1* 5′ upstream region and 176 bp of the *wun1* 5′ untranslated region were fused to CAT. As a 3′ end, the terminator sequence of the 35S gene from cauliflower mosaic virus was used (Töpfer et al., 1987).

Essentially the same transcriptional fusion was constructed with *wun1*-NPTII except for the termination signal. In this case the 3′ end was derived from the octopine-synthase gene (B. Reiss, personal communication).

Analysis of *wun1* Promoter Activity by Transient Expression of *wun1*-CAT DNA in Potato and Tobacco Protoplasts

To get an insight into the functionality of the *wun1* promoter, a chimeric construct *wun1*-CAT was tested by transient expression experiments with potato protoplasts. A precondition for the activity of the *wun1* promoter in a transient potato system must be the presence of corresponding *trans*-acting factors capable of driving the endogenous *wun1* gene. As shown in Figure 2, *wun1* mRNA was detected in potato suspension cultures (SC) as well as in suspension-derived protoplasts. Control hybridiza-

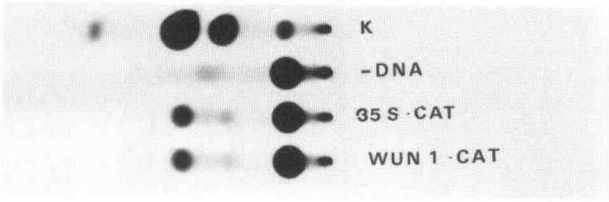

Figure 3. *wun1*-CAT Is Active Transiently in Potato Protoplasts.

CAT assays were performed from transient expression experiments using 20 μg of plasmid DNA per 1×10^6 potato protoplasts. 35S-CAT = pRT101-CAT, a plasmid that contains the 35S promoter in front of the CAT gene; −DNA = 1×10^6 protoplasts treated like the others but without adding DNA; K = the CAT activity of 1 unit of chloramphenicol acyltransferase.

tions with the cDNA of another potato wound-inducible gene (*wun2*) (Logemann et al., 1988) gave similar results. In contrast, a potato tuber-derived patatin cDNA probe did not detect any mRNA in potato suspension cultures or in protoplasts.

To characterize the strength of the *wun1* promoter, we compared CAT activities resulting from transient expression driven by the 35S promoter with those obtained with the *wun1* promoter in the same protoplast system. Interestingly, the activity of 35S-CAT was only slightly higher than that of *wun1*-CAT (Figure 3).

Although tobacco mRNA hybridizes only weakly to a *wun1* probe, the *wun1*-CAT gene was found to be expressed well in tobacco protoplasts (data not shown).

Analysis of *wun1*-CAT Constructs in Transgenic Tobacco Plants

To complement the data on *wun1*-CAT expression in potato and tobacco protoplasts, transgenic *wun1*-CAT tobacco plants were obtained using the *Agrobacterium tumefaciens* 3850_{KM} vector system (Jones et al., 1985). Nopalin-positive and kanamycin-resistant LS2 tobacco plants were shown to contain between two and four unrearranged T-DNA copies by DNA gel blot hybridization and subsequently were grown in the greenhouse. In Figure 4, CAT activities of three independent transformed tobacco plants are shown. Mechanical wounding of leaves, as described in "Methods," led to a CAT activity that was about 4 times higher than in nonwounded leaves. When an extract derived from protoplasts tubers (see "Methods") was added to wounded tobacco leaves, CAT activity was apparently 6 times higher, compared with nonwounded leaves. Extracts from wounded and nonwounded tubers taken either directly from the plant or from stored tubers were used in these experiments. In all cases the increase in CAT activity was similar (Figure 5), which suggests that a fairly stable *wun1*-stimulating factor is present in potato tubers prior to wounding.

Interestingly, the wound inducibility of *wun1*-CAT in LS2 plants was more pronounced in plants grown under sterile conditions. Seeds from selved LS2 plants were harvested and grown in sterile culture under kanamycin selection. The comparison of the CAT activities of greenhouse- and sterile culture-derived LS2 plants demonstrated clearly that, in sterile culture, the CAT activity of young nonwounded leaves was lower. Wounding of these leaves led to 40 times more CAT activity. The addition of tuber extracts to the wounding assay increased the CAT activity 60-fold (data not shown).

Transient Expression Experiments Using Protoplasts from Several Plant Species Including Rice

Transient expression experiments are a convenient way to test the functionality of a promoter construct. In addition, they indicate in which plant species foreign promoter activity can be expected.

For the following experiments, a *wun1*-NPTII construct was used containing the same *wun1* promoter fragment as *wun1*-CAT. *wun1*-NPTII-derived NPTII activity was observed in potato, tobacco, and parsley protoplasts (data not shown). A very interesting result was obtained with protoplasts from rice. Using *wun1*-NPTII DNA in two independent experiments, the activity of the *wun1* promoter in rice protoplasts was as high as in a potato protoplast system (Figure 6). No *wun1* cross-hybridizing DNA or RNA was detected in that monocot plant (data not shown).

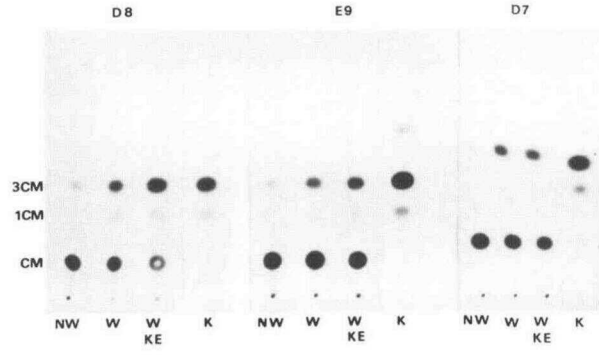

Figure 4. Influence of Wounding on the CAT Activity of Transgenic LS2 Leaves.

CAT assays of leaf extracts of three transgenic LS2 plants (D8, E9, D7) were performed. NW = nonwounded leaves; W = wounded leaves; WKE = wounded leaves that were incubated additionally with a potato tuber extract derived from fresh, wounded tubers (see "Methods"); K = the CAT activity of 1 unit of chloramphenicol acyltransferase; CM = chloramphenicol; 1CM = 1-acetyl chloramphenicol; 3CM = 3-acetyl chloramphenicol.

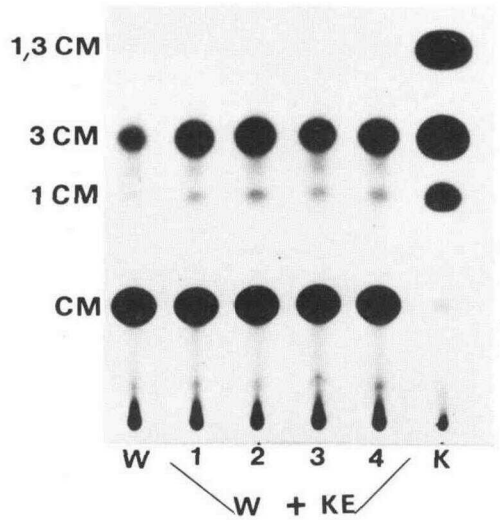

Figure 5. Addition of Tuber Extracts Increases the *wun1* Promoter Activity in Wounded LS2 Leaves.

CAT assays were performed using extracts from wounded (W) or from wounded and additionally incubated with different potato tuber extracts (W + KE 1 to 4) leaves of LS2-D7. 1 = extract taken from fresh tubers that were wounded; 2 = same as 1, but without wounding; 3 = extract taken from tubers that were stored for 4 months at 10°C and then wounded; 4 = same as 3, but without wounding; K = the CAT activity of 1 unit of chloramphenicol acyltransferase; CM = chloramphenicol; 1CM = 1-acetyl chloramphenicol; 3CM = 3-acetyl chloramphenicol; 13CM = 1,3-diacetyl chloramphenicol.

Expression of Endogenous *wun1* mRNA in *Phytophthora*-Infected Potato Leaves

To test whether the wound-inducible gene *wun1* behaves as a pathogenesis-related gene, we studied its possible induction by pathogen infection. Leaves of the potato variety Datura were cut at their bases and the bases were soaked in water. Three different solutions subsequently were sprayed onto their leaves:

- A suspension containing fungal spores of the pathogen *P. infestans* of the variety Pi1 that are compatible with Datura plants.
- A suspension containing *P. infestans* spores of the variety Pi4 that are imcompatible with Datura plants.
- Cut off leaves sprayed with water devoid of spores (as a control).

After different times, mRNA was isolated and used for RNA gel blot analysis. The intensity of hybridization of a *wun1* cDNA probe was measured by a densitometer and documented by the curves shown in Figure 7. As soon as 2 hr after spray treatment, the amount of *wun1* mRNA increased 4-fold and remained more or less at this level for the next 8 hr. This result was obtained independently of whether water alone, or the compatible Pi1 strain, or the incompatible Pi4 strain was used for spraying. About 12 hr after treatment, significant differences became obvious between leaves infected with either Pi1 or Pi4. Although the *wun1* mRNA expression leveled off after 10 hr in potato leaves sprayed with water or with a Pi4 spore suspension, this was not the case with Pi1-infected leaves, in which *wun1* mRNA levels continued to increase with time.

Whereas Pi4-treated leaves only showed epidermal hypersensitivity spots 16 hr after fungal infection, Pi1-treated leaves started to show collapse of mesophyll cells within this time. A correlation between the level of *wun1* mRNA accumulation and the degree of leaf destruction is obvious. The use of other cDNA probes either from another wound-inducible potato gene, called *wun2* (Logemann et al., 1988), or from the proteinase inhibitor II from potato (Sanchez-Serrano et al., 1986), or of patatin (Rosahl et al., 1986) did not lead to any significant hybridization (data not shown).

DISCUSSION

wun1 (Logemann et al., 1988) is a gene that is rapidly induced by wounding in most organs of potato (tubers, leaves, stems, and roots) (J. Logemann and J. Schell, manuscript in preparation). The function of the product of this gene is still unknown but shows some similarities with

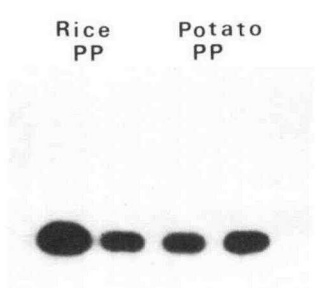

Figure 6. Transient NPT Activity of *wun1*-NPTII in Rice and Potato Protoplasts.

In two independent experiments, 20 μg of *wun1*-NPTII DNA (see Figure 1) were used for transient expression experiments with 1 $\times$ 10^6 rice protoplasts (Rice PP) and potato protoplasts (Potato PP). NPTII activity was monitored as described by Reiss et al. (1984).

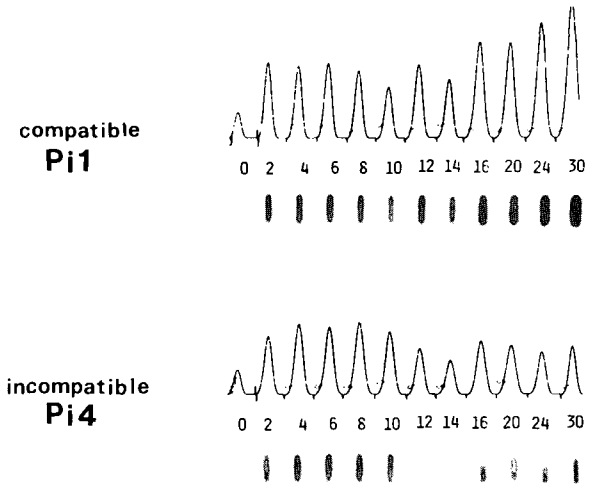

Figure 7. Infection of Potato Leaves with Different *Phytophthora* Strains Led to Differences in *wun1* mRNA Accumulation.

Ten μg of total RNA derived from potato leaves were tested in an RNA gel blot experiment. These leaves were infected with water containing spores of either compatible (compatible, Pi1) or incompatible (incompatible, Pi4) *P. infestans* strains. The numbers indicate the time point of RNA isolation after infection. The hybridization pattern against a *wun1* cDNA probe is shown and indicated additionally by curves derived from densitometric measurement of the autoradiographs. The results obtained in control leaves sprayed with water devoid of fungal spores were essentially the same as those obtained with Pi4 spores and are not shown.

so-called pathogenesis-related proteins (Van Loon, 1985). Indeed *wun1* codes for a small protein (18 kD, Logemann et al., 1988) and accumulates rapidly as a result of wounding. In addition, a striking accumulation of *wun1* mRNA was observed in potato leaves collapsing as a result of fungal infection. Whereas in cut leaves infected with the incompatible Pi4 strain of *P. infestans wun1* mRNA accumulation leveled off 10 to 12 hr after infection, it continued to accumulate in leaves infected with the compatible spores Pi1 at a time when these infected tissues were collapsing (16 to 30 hr after infection; see Figure 7). Therefore, the *wun1* product might be involved in a general physiological reaction to stresses correlated with cell death and not in a more specific disease resistance.

cis-Acting elements involved in the regulated expression of *wun1* were shown to be contained entirely within a 1.2-kb fragment derived from the immediate 5′ upstream region of *wun1*. This was shown by the construction of *wun1*-CAT reporter genes, the expression of which was analyzed in stable transgenic tobacco plants and transiently in protoplasts derived from potato, tobacco, parsley, and rice. Although a *wun1* cDNA probe hybridizes only weakly with tobacco mRNA, tobacco leaves must, nevertheless, contain the necessary *trans*-acting factors to induce a *wun1*-CAT gene after wounding. In fact, a 60-fold induction of CAT activity was detected upon wounding sterile tobacco leaves. Leaves from greenhouse-grown tobacco plants that were subjected to infection with several pathogens showed a much higher level of CAT activity in nonwounded leaves, indicating that the *wun1* promoter is activated probably by pathogen stress in tobacco leaves. The effect of wounding on the activation of transcription of *wun1* is mediated probably by a factor. This view is supported by the fact that extracts from wounded potato tubers stimulate *wun1*-CAT induction in transgenic tobacco leaves. This inducer is, in fact, present latently in nonwounded potato tubers and is heat-resistant, since boiling does not destroy its activity. This factor might, therefore, be analogous to the proteinase inhibitor-inducing factor (Bishop et al., 1984).

Transient expression experiments are very useful systems to test promoter functionality in homologous and heterologous plant systems. In the case of inducible genes like *wun1*, promoter activity can be expected only provided that the transient expression system mimics the inducing situation. High expression rates of endogenous *wun1* mRNA in potato suspension cultures and derived protoplasts indeed indicate the presence of *trans*-acting factors that are needed for *wun1* gene expression. Presumably, the culturing of cells in suspension as well as the protoplasting produce a physiological situation that is equivalent to wounding of potato tissue. It appears that the strength of the *wun1* promoter is comparable to that of the ^{35}S promoter. This observation was confirmed by preliminary results measuring NPTII activities in ^{35}S-NPTII transgenic tobacco leaves and in wound-induced *wun1*-NPTII transgenic tobacco leaves (data not shown).

To test inducibility of the *wun1* promoter in transient expression experiments, we tried to find agents that would further stimulate *wun1* expression over a background level. The addition of several agents that are known to stimulate wound-induced genes such as polygalacturonic acid, chitosan (Walker-Simmons et al., 1983), or potato tuber extracts (see "Results") to potato protoplasts did not result in any detectable change in the level of expression of *wun1*-CAT genes or of the endogenous *wun1* gene (data not shown). Probably the maximum level of expression is already reached so that no further stimulation is possible.

Although no *wun1* cross-hybridizing RNA can be detected in parsley and rice protoplasts, transient expression of *wun1*-CAT and *wun1*-NPT constructs was achieved readily in protoplasts derived from these species. In rice protoplasts, the *wun1* promoter activity was comparable in strength to its activity in potato protoplasts. Obviously, the *trans*-acting factors that are necessary for *wun1* expression are conserved in both monocot and dicot plants.

METHODS

Clones, Bacteria, and Plant Material

^{32}P-labeled radioactive probes for RNA gel blot hybridization were the cDNA clones *wun1*-25A2, *wun2*-29C12 (Logemann et al., 1988), and patatin (pcT1500, Rosahl et al., 1986). Experiments with potato leaves were done with a tetraploid commercial variety of *Solanum tuberosum* (Datura). For stable transformation, the tobacco variety Samsum NN (LS2) was used. Transient expression experiments were performed by isolating protoplasts from suspension cultures of the following plants: explants of potato stems of the commercial variety Datura (D12), tobacco SR1 shoots, parsley suspension cultures (*Petroselinum hortense*), and rice suspension cultures (*Oryza sativa japonica* cv Taipei). The plasmid pRT101 that contains a ^{35}S-CAT construct was used for transient expression experiments in potato protoplasts and was a gift of M. Pröls (Pröls et al., 1988).

Recombinant DNA Techniques

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

Isolation and Analysis of Nucleic Acids

Isolation of DNA from Agrobacteria as well as from higher plants was carried out according to Murray and Thompson (1980). Total RNA was isolated from plants, suspension cultures, and protoplasts according to Logemann et al., (1987). The analyses of DNA and RNA by blot hybridization were performed according to Eckes et al., (1985).

Transfer of *wun1*-Derived Constructs into Tobacco Plants

The chimeric gene *wun1*-CAT present in the intermediate vector pMPK110 (Eckes et al., 1986) was transferred into the T-DNA of the disarmed strain *Agrobacterium* C58C1 (pGV 3850 Kan^R) (Zambryski et al., 1983; Jones et al., 1985) by a triparental mating using pGJ 23 as the mobilizing strain (Van Haute et al., 1983). Co-integrates were selected in the presence of spectinomycin (100 μg/ml), chloramphenicol (25 μg/ml), streptomycin (300 μg/ml), and erythromycin (50 μg/ml). Agrobacteria growing in the presence of these antibiotics were checked subsequently for the presence of a nonrearranged T region and used for transformation of tobacco leaf discs (Horsch et al., 1985). Shoots growing on 100 μg/ml kanamycin were rooted on hormone-free medium containing the same amount of kanamycin and tested subsequently for nopaline synthase activity (Otten and Schilperoort, 1978). Only kanamycin-resistant and nopaline synthase-positive plants were transferred to the greenhouse and cultivated further.

All manipulations were performed according to standards of the Bundesministerium für Forschung und Technologie.

Wounding Experiments

Nonwounded tobacco leaves and potato tubers were cut from the plant and frozen immediately in liquid nitrogen. The wounding of tobacco leaves was performed by slicing them into small pieces and incubating them in phosphate solution (20 mM sodium phosphate, pH 7.0, rifampicin (100 μg/ml)) for 18 hr at 28°C in the dark. Potato tubers were cut into 3-mm pieces and incubated under the same conditions. Afterward, the material was stored at −70°C.

Preparation and Application of Potato Tuber Extracts

Fresh tubers (taken directly from the plant) or stored tubers (stored 4 months at around 10°C) were used. For preparing extracts, the tubers were homogenized at 4°C without adding any buffer. After 10 min of centrifugation at 10,000 rpm in an SS-34 rotor, the supernatant was isolated and boiled for 10 min. After short centrifugation, the clear supernatant was mixed with 20 mM phosphate buffer, pH 7.0 (1:10, v:v). Wounded plant tissue was incubated in this solution for 18 hr at 28°C in the dark.

Preparation and Application of PGA or Chitosan

PGA and chitosan were prepared as described (Walker-Simmons et al., 1983) and used in concentrations of 0.2% PGA and 2.0% chitosan.

Infection of Potato Leaves with *Phytophthora infestans*

Infection of potato leaves with Pi1 and Pi4 spores was performed as described by Cuypers and Hahlbrock (1988) except for the fact that the bases of the cut off and infected leaves were submerged in water.

Transient Expression Experiments

For potato protoplasts, potato suspension cells of stem explants of the variety Datura 12 were collected by centrifugation and incubated in 2% cellulase RS (Onozuka) 0.5% mazerozym in B5 (GIBCO) + 14% sucrose for 12 hr (pH 5.6, 28°C). After enzyme treatment, they were spun for 5 min at 100*g*. The floating protoplasts were taken off and purified further as described by S. Lipphardt, R. Brettschneider, F. Kreuzaler, J. Schell, and J. Dangl, (manuscript submitted to EMBO J.). In each experiment, 10^6 protoplasts were transformed with 20 μg of CsCl-gradient purified DNA.

For rice-protoplasts, 20 μg of highly purified plasmid DNA were used for the transformation of 1×10^6 protoplasts derived from rice suspension of cv. Taipei 309 using the polyethylene glycol-$CaCl_2$ method according to Werr and Lörz (1986) and Junker et al. (1987).

Detection of the Enzymatic Activity of Different Marker Genes

CAT activity was measured according to Velten and Schell (1985). The detection of NPTII activity was described by Reiss et al. (1984).

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