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cis-Analysis of the Wound-Inducible Promoter *wun1* in Transgenic Tobacco Plants and Histochemical Localization of Its Expression

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The 5′ region of the wound-inducible gene *wun1*, derived from potato, has been sequenced and analyzed for *cis*-acting elements important in controlling gene expression in transgenic tobacco plants. Different 5′ deletion fragments were linked to the reporter gene β-glucuronidase (GUS) as transcriptional fusions, and the expression of these chimeric genes was analyzed in leaf tissue. Sequences 111 base pairs upstream of the transcriptional start site were not able to drive the GUS expression over background levels, whereas sequences between −111 and −571 showed a slightly higher activity with equal levels of transcription in wounded and nonwounded tissue. The addition of further upstream sequences (−571 to −1022) enhanced the level of expression by a factor between 13 and 370. The expression driven by this fragment was inducible by a factor of twofold to ninefold by wounding. Histochemical analysis of different tissue from transgenic plants that contain *wun1*-GUS fusions demonstrates wound-inducible and cell-specific *wun1* promoter activity in plants containing the −1022-base pair fragment. The location of GUS activity appears to be cell-specific, being highest in epidermal cells of leaves and stems and lower in vascular cells. Activity was reduced to levels that could not be detected by histochemical staining in leaves, stems, and roots of plants containing the deleted promoter fragments. Plants that contain the different deletion constructs and plants that carry the −1022-base pair fragment show high expression in anthers and pollen grains that could not be stimulated by wounding.

INTRODUCTION

Wounding caused by physical damage of plant tissue leads to several changes in protein and RNA patterns. Different cDNA clones could be identified whose corresponding mRNA amounts increase as a consequence of wounding (Chen and Varner, 1985; Sanchez-Serrano et al., 1986; Fritzemeier et al., 1987; Ryder et al., 1987; Bohlmann et al., 1988; Kombrink, Schröder, and Hahlbrock, 1988; Vornam, Schön, and Kindl, 1988; Bowler et al., 1989). However, *cis*-analysis of the regulatory regions responsible for wound-specific gene expression is still at a preliminary stage. The proteinase inhibitor II genes of potato and tomato (Sanchez-Serrano et al., 1987; Thornburg et al., 1987), as well as the *wun1* gene from potato (Logemann et al., 1988, 1989), have been characterized to a certain extent. mRNAs corresponding to these genes accumulate in wounded leaves to a considerable level although they are not detectable in nonwounded leaves (Ryan, 1984; Sanchez-Serrano et al., 1987; Logemann et al., 1988). Chimeric genes consisting of the proteinase inhibitor II promoter fused to chloramphenicol acetyltransferase (CAT) as a reporter enzyme showed high and wound-inducible CAT activity in transgenic tobacco plants when the 3′-untranslated region from the proteinase inhibitor II gene was inserted in the construct (Thornburg et al., 1987; An et al., 1989). We have shown previously that a *wun1*-CAT chimeric gene containing 1022 bp of the *wun1* promoter and a poly-A addition sequence from the 35S RNA of the cauliflower mosaic virus was sufficient to increase CAT activity after wounding in transgenic tobacco plants (Logemann et al., 1989). To identify relevant *cis*-acting elements, 5′ deletion fragments of the *wun1* promoter were transcriptionally fused to the β-glucuronidase (GUS) coding sequence and analyzed in transgenic tobacco. A region responsible for wound inducibility and enhancement could be identified. Histochemical staining allowed the detection of wound-inducible as well as constitutive expression of GUS in a cell-specific manner.

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RESULTS

Sequence Analysis of the *wun1* 5′ Region

1201 bp of a genomic *wun1* fragment derived from the haploid potato line AM80/5793 were sequenced. Along with the transcriptional start site (J. Logemann and J. Schell, manuscript submitted), this (EcoRI-XhoI) fragment contains 1022 bp of the *wun1* 5′ upstream region and 178 bp of its 5′-untranslated region. As indicated in Figure 1, typical sequence elements like TATA (−33 bp) and CAAT box (−58 bp) are present. Several direct and inverted repeats are located in the upstream region of the promoter fragment. Seven inverted repeats with an average size of 30 bp and two direct repeats of 18 bp and 20 bp, respectively, are located between position −1022 and −509. A striking array with 16 repeated AT pairs is located at −949 to −917 and creates an inverted repeat with 100% homology. The homology of the other two direct and six inverted repeats shown in Figure 1 varies between 65% and 100%.

5′ Deletion Analysis of the *wun1* Promoter in Transgenic Tobacco

It was shown previously (Logemann et al., 1989) that a *wun1*-CAT chimeric gene consisting of 1022 bp of the *wun1* 5′ region is sufficient for high and wound-inducible gene expression in transgenic tobacco leaves. To identify potential *cis*-acting regulatory regions, the complete 5′ region of *wun1* (pLS034-1022) and three 5′ deletion fragments were fused transcriptionally with GUS and designated pLS034-571, pLS034-111, and pLS034-86, as shown in Figure 2B. As a control, the 5′-flanking region up to −1022 was fused in the reverse orientation to GUS (pLS034-1022R). All constructs were cloned into pPR69, a derivative of the binary vector Bin19 (Bevan, 1984; Jefferson, 1987), and transferred to tobacco (var. W38) via *Agrobacterium tumefaciens* LBA4404 (Hoekema et al., 1983)-mediated transformation. After regeneration, kanamycin-resistant plants were analyzed by DNA gel blot analysis (data not shown). Six to 12 independent transformants of each construct were grown in the greenhouse and tested for GUS activity in wounded and nonwounded leaves. Untransformed tobacco leaves (W38) were taken as a control (K). Transgenic leaves containing pLS034-86 and pLS034-111, as well as the reversed promoter fragment (pLS034-1022R), showed very low levels of GUS activity comparable with the activity of untransformed tobacco leaves, as illustrated in Figure 3. These data were obtained independently of whether the GUS activity of leaves was measured under wounding or nonwounding conditions. A fourfold higher expression was found when sequences between −111 bp and −571 bp (pLS034-571)

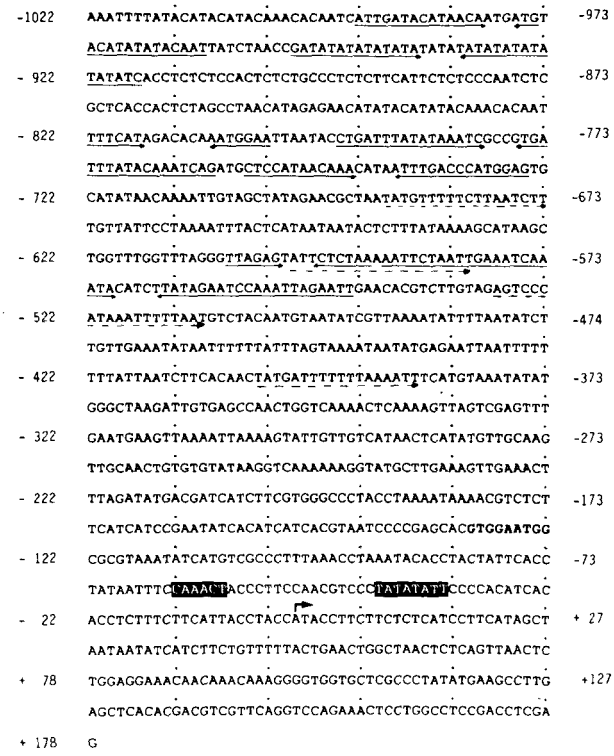


Figure 1. DNA Sequence of the 5′ Upstream Region of the *wun1* Gene.

Numbering depends on the determined transcriptional start site (Logemann et al., 1988). The TATA and CAAT box are indicated by boxes. The transcription start point is marked by an arrow. Direct and inverted repeats are underlined (– – ⟶, ⟶ ⟵).

were used, but this expression was not affected by wounding. The addition of a further 451 bp (pLS034-1022) led to a 13-fold to 370-fold increase in GUS expression with respect to pLS034-86. This observation was made with wounded leaves, whereas nonwounded leaves showed a twofold to ninefold lower activity.

Histochemical in Situ Analysis of Transgenic Plants

To determine the cell-specific or tissue-specific expression of *wun1*-GUS in transgenic tobacco plants, a histochemical method for the identification of GUS expression was used (Jefferson, 1987). This method includes the overnight staining of tissue sections with *X*-Gluc (5-bromo-4-chloro-3-indolyl glucuronide), and, therefore, shares some similarity to the wounding conditions described (see Methods). To distinguish GUS activity in wounded and nonwounded tissue, the following treatment was chosen: (1) for wounding conditions, tissue was cut in slices and incubated for 18 hr in phosphate buffer prior to overnight staining in *X*-

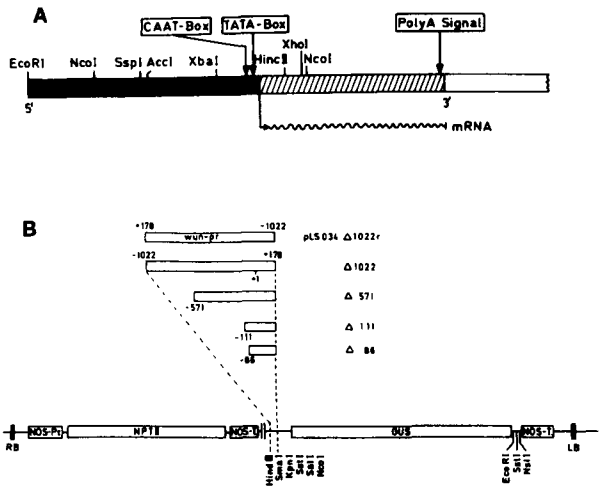


Figure 2. Organization of the Genomic Clone *wun1* and Construction of Vectors Containing Different *wun1* 5′ Deletions.

(A) Restriction map of the genomic clone *wun1*-85. The region between EcoRI and XhoI was sequenced in both directions. It contains 1022 bp of the promoter region (black box) and 178 bp of the 5′-untranslated region, which is part of the 217-bp *wun1* 5′-untranslated region. Regulatory sequences are indicated by white boxes.

(B) Construction of chimeric genes carrying different 5′ deletion fragments of *wun1*. In between the left and right T-DNA borders of pLS034, an NPT II gene is present under control of the *nos* promoter for selection on kanamycin. The GUS gene carrying a *nos* poly-A addition sequence is under control of different 5′ upstream deletion fragments of the *wun1* gene. The end positions of the 5′ upstream fragments are given as numbers beside the different constructs. R, cloning of the fragments in the reversed orientation. The plasmid pLS034-1022 contains all restriction sites of the M13mp18 polylinker (HindIII to EcoRI) in front of the *wun1* promoter. Deletion clones were obtained by cutting with SphI and XbaI before exonuclease digestion. The distance between the *nos* terminator of the NPT II gene and the *wun1* 5′ region is about 1 kb.

Gluc solution (see Methods); (2) for nonwounding conditions, tissue was cut and incubated in *X*-Gluc solution containing 1.8 mM cycloheximide (Pröls et al., 1988) as a translational inhibitor and stained in an overnight reaction. This procedure was used to inhibit the induced expression of GUS due to the staining conditions. The activity of the enzyme β-glucuronidase, which is already present in unwounded tissue, is not affected by this inhibitor (data not shown). All plants carrying the *wun1* promoter fragments pLS034-571, pLS034-111, and pLS034-86, as well as pLS034-1201R, did not show any detectable staining of leaf tissue independent of wounding (data not shown). The same result was observed with leaves from untransformed tobacco plants. Leaves of wounded plants carrying the whole *wun1* promoter fragment (pLS034-1022 bp) showed a specific staining of epidermal cells (including trichomes) of leaf midribs and blades, as illustrated in Figure 4. This staining also appeared in several cell layers beneath the epidermis but became weaker in the inner parts. Strongly expressing plants showed additional staining of the vascular system. Significant differences in GUS expression could be observed in cross-sections of wounded or cycloheximide-treated leaves. In leaves treated with cycloheximide, staining was usually absent. In some cases, only epidermal cells of the region that protruded from the midrib to the leaf blades were stained (Figure 4B). In wounded stems of pLS034-1022 transgenic plants, mostly the outer

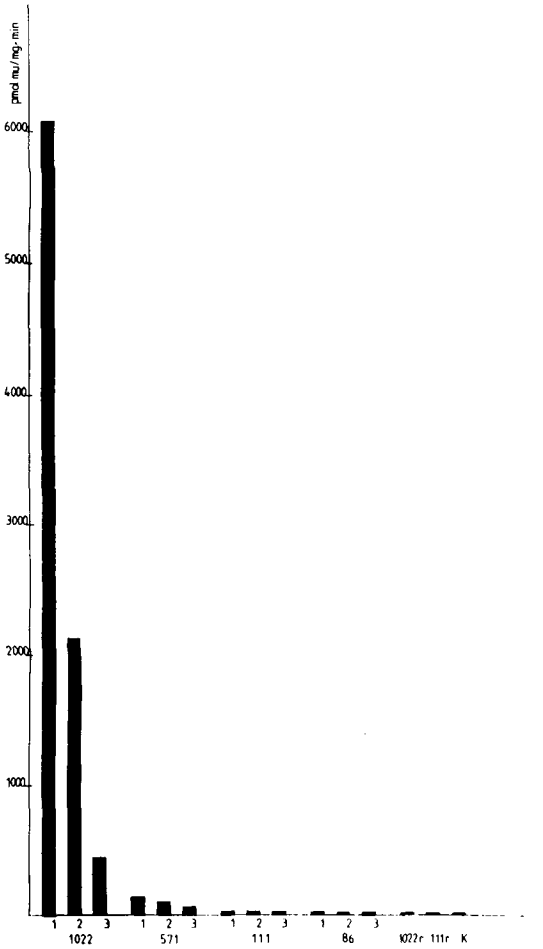
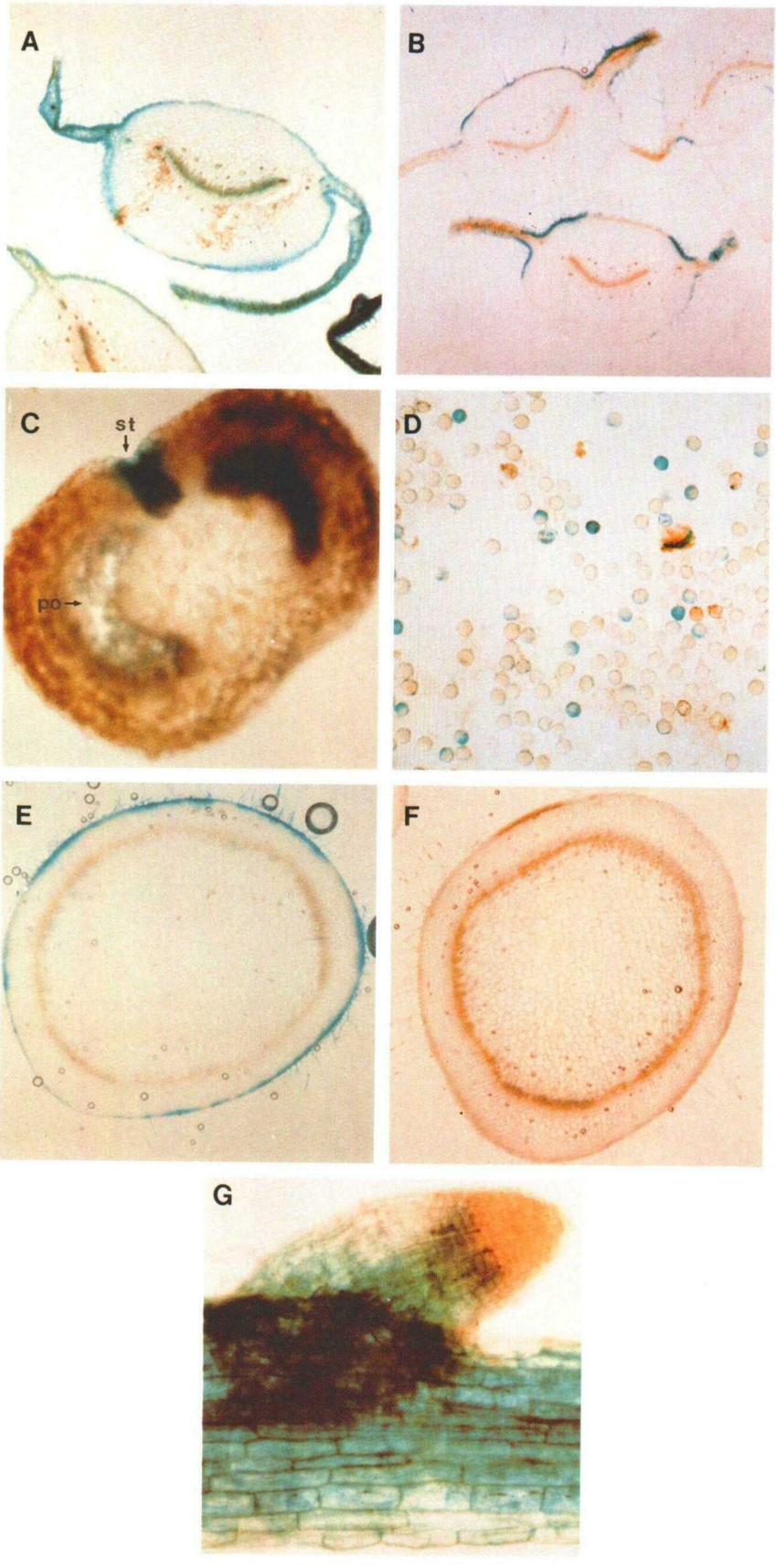


Figure 3. GUS Activity Analysis of Tobacco Carrying Different *wun1* 5′ Deletion Fragments.

Transgenic tobacco leaves from the greenhouse were tested for GUS activity depending on different 5′ upstream fragments of the *wun1* gene. The enzyme activity of GUS is shown as picomoles per milligram of protein per minute on the abscissa. The ordinate shows three out of six to 12 plants tested. These three plants reflect the highest, lowest, and average results of *wun1* promoter activity of all plants tested after wounding.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epidermis stained to an intense blue (Figure 4E), whereas in cycloheximide-treated stems, a very weak blue staining was present in the epidermis (Figure 4F). Wounded root pieces incubated in X-Gluc stained to an intense overall blue with the exception of the root tip area, where the meristem for growth and the calyptra reside. The same pattern could be observed in root pieces treated with cycloheximide, although the intensity of staining was lower (data not shown).

Transgenic plants carrying any deletion of the full-length promoter (pLS034-571, pLS034-111, pLS034-86) or carrying the inverted promoter fragment (pLS034-1022R) did not show any detectable staining of leaves, stems, or roots independently of whether the tissue had been wounded or treated with cycloheximide (see Table 1). In addition, untransformed plants showed no GUS activity. To detect GUS activity in flowers, cross-sections were stained with X-Gluc. In plants containing the complete *wun1* 5′ region (pLS034-1022), the stomium of anthers and 30% of the pollen grains were stained blue (Figure 4C). There were no differences in the staining of wounded or cycloheximide-treated anthers. This observation was reinforced by measuring GUS activity of wounded and nonwounded anthers. There was no significant change in GUS activity after wounding (data not shown), indicating a constitutive expression in this tissue. Anthers derived from pLS034-111 or pLS034-1022R, as well as untransformed plants (see also Plegt and Bino, 1989), did not show any staining of the stomium even after wounding. Moreover, the amount of stained pollen grains was reduced to about 0.75%. In contrast, plants carrying pLS034-571 or pLS034-86 showed the same staining pattern of the stomium and pollen grains as with the complete *wun1* promoter fragment (pLS034-1022). The only difference was the intensity of stained tissue, which was weaker than in plants carrying pLS034-1022. Further analysis by GUS measurements showed 7 times weaker GUS activity in anthers from pLS034-86 plants than in pLS034-1022 plants.

Table 1. Comparison of GUS activity in Wounded and Nonwounded Tissue of Transgenic Plants Carrying Different *wun1* 5′ Deletions

5′ Region	−1022	−571	−111	−86	−1022RR
Tissue					
Leaf	Ind.[a]	–[b]	–	–	–
Stem	Ind.	–	–	–	–
Root	Ind.	–	–	–	–
Anther	Const.[c]	Const.	–	Const.	–
Pollen	Const.	Const.	–	Const.	–

[a] Ind., inducible GUS activity. In wounded tissue, the GUS activity is significantly higher than in nonwounded tissue.
[b] –, no significant GUS activity, with or without wounding.
[c] Const., constitutive GUS activity. In wounded tissue, the GUS activity is the same as in nonwounded tissue.

DISCUSSION

A functional analysis of the *wun1* 5′ upstream was carried out in transgenic tobacco plants. The sequence of the tested *wun1* full-length promoter comprised 1201 bp, including 178 bp of the *wun1* 5′-untranslated region. Several 5′ deletion mutants were fused to GUS and were tested in transgenic tobacco plants. A region responsible for wound inducibility and enhancement of this expression could be identified. Sequences up to −111, including TATA and CAAT box, showed only background expression level in transgenic leaves that could not be further stimulated by wounding. The addition of upstream sequences (pLS034-571) demonstrated a fourfold higher but still very low GUS expression in nonwounded and in wounded leaves. The addition of further 451 bp (pLS034-1022) enhanced the overall expression 13-fold to 370-fold and introduced wound inducibility twofold to ninefold. To find homologies to other genes involved in wound response, the sequence of the *wun1* gene was compared with that of the *win1* gene (Stanford, Bevan, and Northcote, 1989). *win1* is also known to be wound-inducible in potato plants. A comparison between the 5′-flanking region of both genes showed two boxes of 85% and 71% homology, respectively, at position −470 to −423 of the *wun1* promoter, as illustrated in Figure 5. Whether these boxes play a role in wound inducibility will have to be proven. Deletion clones of the *wun1* 5′ region that contain this sequence (pLS034-571) and no further upstream sequences show no wound-inducible expression. Further sequence comparison with other promoters involved in wound response

Figure 4. Cell-Specific Expression of β-Glucuronidase in Transgenic Tobacco Plants Stained with X-Gluc.

(A) to **(F)** Cross-sections of transgenic tobacco plants carrying pLS034-1022.
(A) Cross-section of a wounded leaf (see Methods).
(B) Cross-section of a nonwounded leaf (see Methods).
(C) Cross-section of an anther. st, stomium; po, pollen grains.
(D) Pollen grains.
(E) Wounded stem.
(F) Nonwounded stem.
(G) Root pieces incubated in X-Gluc are blue all over except for the root tip.

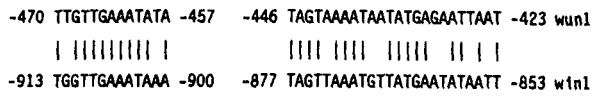

Figure 5. Sequence Comparison between Two Wound-Inducible Genes from Potato.

Sequence comparison of the genomic clones *wun1* and *win1* derived from potato show two boxes of high homology (85% and 71%, respectively). The numbers given in front and behind the sequences indicate the position relative to the transcription start point (+1).

or stress response [for example, the proteinase inhibitor II gene from potato (Sanchez-Serrano et al., 1987)] revealed no significant homologies.

The histochemical analysis of transgenic tobacco plants carrying *wun1*-GUS deletion mutants confirms the results taken from GUS activity measurements. Leaves, stems, and roots of transgenic plants carrying the whole *wun1* promoter fragment (pLS034-1022) show high and wound-inducible GUS activity. In *X*-Gluc-stained cross-sections of these tissues, the GUS activity could be specifically located in the epidermis of leaves and stems. According to the method of Jefferson (1987), the incubation of cross-sections with *X*-Gluc needs several hours until a blue staining appears. Because the staining conditions mimic wounding, activity of the *wun1* promoter in nonwounded tissue was visualized with the addition of cycloheximide, a translational inhibitor. This inhibits translation of newly induced GUS mRNA. It was previously demonstrated that the activity of the enzyme β-glucuronidase is not affected by cycloheximide (data not shown). Furthermore, it was shown that GUS activity of nonwounded leaves (tissue directly frozen after cutting) of transgenic *wun1*-GUS plants (pLS034-1022) was the same as in leaves that were treated with cycloheximide (data not shown). Therefore, the application of cycloheximide allows us to visualize gene activity as present prior to wounding.

The high and specific expression of *wun1* in the outer cells of leaves and stems emphasizes the hypothesis that *wun1* might be involved in the defense of pathogens that invade the plant mainly through the epidermis. Indeed, similarities between cells containing high GUS activity and cells producing callose have been found (J. Logemann and J. Schell, manuscript submitted). Enzymes involved in callose formation as well as suberization and lignification are localized in the epidermis and are involved in pathogen defense mechanisms by producing physical barriers (Hahlbrock and Scheel, 1987). Although the full-length promoter of *wun1* is wound-inducible in several tissues, its expression appears to be constitutive in the stomium and pollen grains of anthers. The function of the stomium is to break open at a certain stage of anther development and to release the pollen grains. Therefore, it is programmed to be destroyed, and one can imagine that a gene that is involved in wounding is constitutively expressed in that area. An analogous expression pattern is known for the thionin gene from barley that appears to be involved in protection against fungal pathogens (Bohlmann et al., 1988). Although thionin is wound-inducible in leaves, it is constitutively expressed in germinating seeds. The working hypothesis is that the chance for a pathogen attack in the early stages of development is so high that a constitutive expression of this toxin makes sense. Histochemical analysis of leaves, stems, and roots of tobacco plants carrying *wun1* promoter fragments smaller than pLS034-1022 did not show any staining. Even leaf cross-sections from pLS034-571 plants were negative to the naked eye, although a slight GUS activity could be measured.

Obviously, staining is less sensitive than the measurement of GUS activity. Staining of stomium and pollen grains led to a different result. With one exception, all deletion fragments carrying the *wun1* promoter in the right orientation led to a blue staining of the stomium, whereas the promoter fragment with reversed orientation did not show any staining. The same observation was made with pollen grains, 30% of which were stained blue in contrast to control plants (below 1% blue) or plants containing the *wun1* promoter in reversed orientation (below 1% blue). With respect to the used promoter fragments, this would mean that a region between −86 and +178 is sufficient for GUS expression in the stomium and pollen grains. A sequence between −86 and −111 reduces the expression in pollen grains and may act as a silencer fragment. Obviously, at least two regulatory regions within the *wun1* promoter do exist to control the expression of this gene in different tissues. It will be of interest to test whether regulatory sequences are located in the uncommonly large (178 bp) 5′-untranslated region of *wun1*.

METHODS

Cloning and Sequencing

The plasmid pLS000 carries a 4-kb genomic fragment containing *wun1*, derived from a genomic library of the haploid potato line AM80/5793 (Logemann et al., 1989). Subcloning of the 5′ fragment in M13mp18 was performed with standard methods (Maniatis, Fritsch, and Sambrook, 1982). Deletion clones were created using exonuclease III and nuclease S1 (Henikoff, 1984). Single-stranded DNA of M13 was sequenced by the chain termination method (Sanger, Nicklen, and Coulson, 1977).

Transformation of Plants

5′ Deletion fragments were cloned upstream of the β-glucuronidase gene of the binary vector pPR69, a derivative of Bin19

(Bevan, 1984; Jefferson, 1987), which was kindly provided by Pascal Ratet and Frans de Bruin. Using a triparental mating procedure with the helper plasmid pRK2013 (Ditta et al., 1980), the pPR69 vector was transferred to the disarmed *Agrobacterium tumefaciens* strain LBA4404 (Hoekema et al., 1983). Colonies of *A. tumefaciens* were tested with a minipreparation of plasmid DNA (Ebert, Ha, and An, 1987). Positive clones were used to infect leaf discs of *Nicotiana tabacum* (var. W38) according to Horsch et al. (1985). Transformants were selected on 50 μg/mL kanamycin and transferred to the greenhouse.

DNA Gel Blot Analysis of Genomic DNA

Genomic DNA was isolated according to the method of Murray and Thompson (1980). DNA was cut with EcoRI and HindIII, fractionated by agarose gel electrophoresis, and tested by DNA gel blot analysis (Southern, 1975) using the GUS coding region as a radioactive labeled probe for hybridization.

GUS Fluorometric Assay of Wounded or Nonwounded Tissue

For nonwounding conditions, leaves were cut from the plant, immediately frozen in liquid nitrogen, and stored at −70°C until use. For wounding conditions, leaves were cut into small pieces and incubated in phosphate buffer (20 mM NaH_2PO_4, pH 7.2, containing Claforan (500 mg/L) for 18 hr at 28°C in the dark. Leaf tissue was tested after incubation or stored at −70°C until use (Logemann, Schell, and Willmitzer, 1987). The protein content of leaf tissue was measured as described by Bradford (1976). The GUS fluorometric assay was done according to Jefferson (1987), except that the assay was carried out in a reaction volume of 100 μL at 37°C. At different time points, starting at zero time, aliquots of 20 μL were removed, and the reaction was stopped by the addition of 980 μL of 0.2 M Na_2CO_3. Fluorescence was measured on a Perkin Elmer LS-2B filter fluorimeter.

Histochemical Staining of Wounded or Cycloheximide-Treated Tissue

The GUS histochemical assay was carried out with the substrate 5-bromo-4-chloro-3-indolyl glucuronide (*X*-Gluc).

Wounded Tissue

Thin sections, cut by hand with a razor blade, were treated as described for wounding conditions for GUS fluorometric assay and incubated in 1 mM to 2 mM *X*-Gluc in 50 mM NaH_2PO_4, pH 7.0, at 37°C overnight, rinsed with 70% ethanol, washed in 50 mM NaH_2PO_4, pH 7.0, and viewed under a microscope.

Cycloheximide-Treated Tissue

Thin sections were incubated in *X*-Gluc solution containing 1.8 mM cycloheximide, a translational inhibitor. Kodak 200 Ektachrome film was used for photography.

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