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Genetics

Differential expression of genes in potato tubers after wounding

(patatin/gene regulation/two-dimensional gel electrophoresis)

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ABSTRACT **Tubers of a common tetraploid species of *Solanum tuberosum* (Granola) were mechanically wounded by slicing. After 18 hr only small differences were detectable in the pattern of the steady-state protein extracted from wounded versus unwounded tubers. In contrast the protein pattern obtained by *in vitro* translation of mRNA isolated from wounded and unwounded tubers differed significantly. A cDNA library was established and screened for wound-induced cDNA clones by differential hybridization. Two clones, wun-1 and wun-2, were found that corresponded to genes that were highly expressed in wounded potato tubers but were not expressed in unwounded tubers. The expression of the gene corresponding to wun-1 is detectable 30 min after wounding; the expression of the gene corresponding to wun-2 is detectable 4 hr after wounding. The expression of both genes (hereafter referred to as wun-1 and wun-2) remains constant for up to 24 hr after wounding. Interestingly the RNA corresponding to patatin, a major storage protein of potato tubers, behaves in the opposite way; it decreases dramatically in tubers within 30 min after wounding. The low level of patatin mRNA observed in unwounded roots and stems also disappears after wounding. Run-off transcription experiments, performed with isolated nuclei, indicate that the activation of the wound-induced genes as well as the inhibition of the patatin gene are controlled at the transcriptional level.**

Mechanically wounding a plant tissue leads to several morphological and physiological changes in the tissue (1). The activity of several enzymes has been shown to increase after wounding, e.g., phenylalanine ammonia lyase and peroxidases in potato tubers (2), extensin in carrot storage tissue (3), fatty acid synthetase in potato tubers (4), and proteinase inhibitor in tomato and potato leaves (5). For some of these enzymes the increase in activity was shown to be correlated with increased mRNA levels (3, 6). One of the best understood wound-induced genes is the proteinase inhibitor from tomato and potato; in wounded leaves the mRNA for this gene is drastically increased (7, 8).

Here we describe the isolation and characterization of two cDNA clones from potato. The expression of the genes corresponding to these cDNAs is induced in tubers after wounding and is transcriptionally regulated. By comparison the expression of patatin in wounded potato tubers was analyzed and found to be regulated in a reciprocal fashion since this gene is switched off by wounding.

MATERIALS AND METHODS

Clones, Bacteria, and Plant Material. The patatin cDNA clone pcT1500 (9) was used to produce labeled hybridization probes. A potato tuber cDNA clone, 25F5, that hybridizes to 25S rRNA was used to standardize RNA amounts in RNA gel blot hybridization and in run off experiments. All RNA gel blot experiments were performed with total RNA. The tetraploid commercial variety of *Solanum tuberosum* (Granola) and the haploid potato genotype AM80/5793 originating from the collection of the Max-Planck-Institut in Cologne were used for the experiments. Unwounded potato leaves, stems, roots, and tubers were cut from a plant growing in the greenhouse and immediately frozen in liquid nitrogen. Potato leaves, stems, roots, and tubers were wounded by slicing them into 3-mm pieces. Slices were incubated in a phosphate solution [20 mM sodium phosphate, pH 7.0, and chloramphenicol (50 μg/ml)] for 18 hr at 28°C in the dark. Potato stems and leaves were cut into small pieces and incubated under the same conditions. Afterwards the material was either used directly or stored at −70°C.

Isolation of RNA. Isolation of total RNA was performed according to Logemann *et al.* (10). Poly(A)$^+$ RNA was isolated by using messenger affinity paper (11).

Construction and Screening of a cDNA Library. Double-stranded cDNA was prepared essentially as described by Gubler and Hoffman (12) starting from poly(A)$^+$ RNA isolated from wounded potato tubers. After second-strand synthesis, the double-stranded cDNA was tailed with cytidines and annealed to a guanosine-tailed *Pst* site in pUC8. *Escherichia coli* strain BMH 71-18 was transformed. Wound-induced cDNA clones were screened by standard differential colony hybridization (13) by using radioactively labeled cDNA from poly(A)$^+$ RNA isolated from either wound-induced or unwounded potato tubers.

Nucleic Acid Isolation and Analysis. Isolation of nuclear, mitochondrial, and chloroplast DNA as well as subsequent analysis of DNA and RNA by blot hybridization was performed according to Eckes *et al.* (14).

Analysis of Transcription in Isolated Nuclei. Isolation of nuclei and *in vitro* transcription were performed according to Willmitzer *et al.* (15). The transcripts were hybridized to cDNAs subcloned in both orientations in M13. To standardize the amount of *in vitro* transcripts, 25F5 cDNA was used as a probe.

Hybrid Selection. Poly(A)$^+$ RNA homologous to clones wun-1 and wun-2 was isolated by selective hybridization by using nitrocellulose filters as described (13).

***In Vitro* Translation of Poly(A)$^+$ RNA.** A commercially available rabbit reticulocyte lysate was used for *in vitro* translation, and the proteins were separated on a denaturing polyacrylamide gel (16). Two-dimensional gel electrophoresis was performed as described (17).

RESULTS

Influence of Wounding on the Pattern of Proteins in Tubers. Tubers of the potato variety Granola were sliced. Unwounded tubers were used as a control. As is obvious from Fig. 1 only minor differences can be detected in the pattern of the steady-state proteins either on denaturing $NaDodSO_4$

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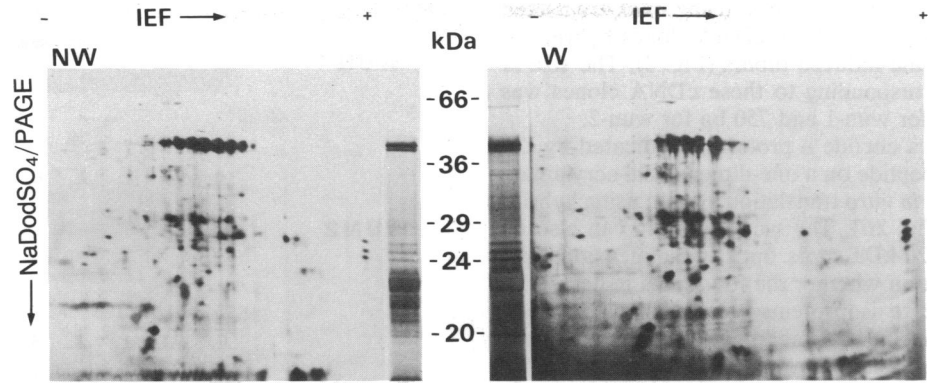

FIG. 1. One- and two-dimensional 15% (wt/vol) acrylamide gel consisting of steady-state proteins isolated from wounded (W) and not wounded (NW) potato tubers. In the first dimension, proteins were separated according to its molecular weight by $NaDodSO_4$/PAGE. The second dimension separates the proteins by isoelectric focusing (IEF).

gels or on two-dimensional gels. This result suggested that at least within 18 hr after wounding the amount of most of the proteins already present in unwounded tubers does not change dramatically. *In vitro* translation of isolated poly-$(A)^+$ RNA from wounded and not wounded tubers, however, led to a significantly different protein pattern on a two-dimensional gel (Fig. 2*A*). Two main differences are seen. (*i*) A 40-kDa major storage protein of potato tubers, called patatin, is present as a group of spots in the translational products derived from unwounded tuber RNA. These spots completely disappear after wounding. (*ii*) Four strong 18- to 20-kDa spots are detected only after wounding.

Isolation of Wound-Induced cDNA Clones. A cDNA library was established with poly$(A)^+$ RNA from wounded tubers. cDNA clones (4000 clones) were screened by differential hybridization with ^{32}P-labeled cDNA preparations from wounded and unwounded tuber tissue as probes. Two wound-specific cDNA clones, subsequently called wun-1 and wun-2, were identified. Further characterization was done by RNA gel blot hybridization to total RNA from

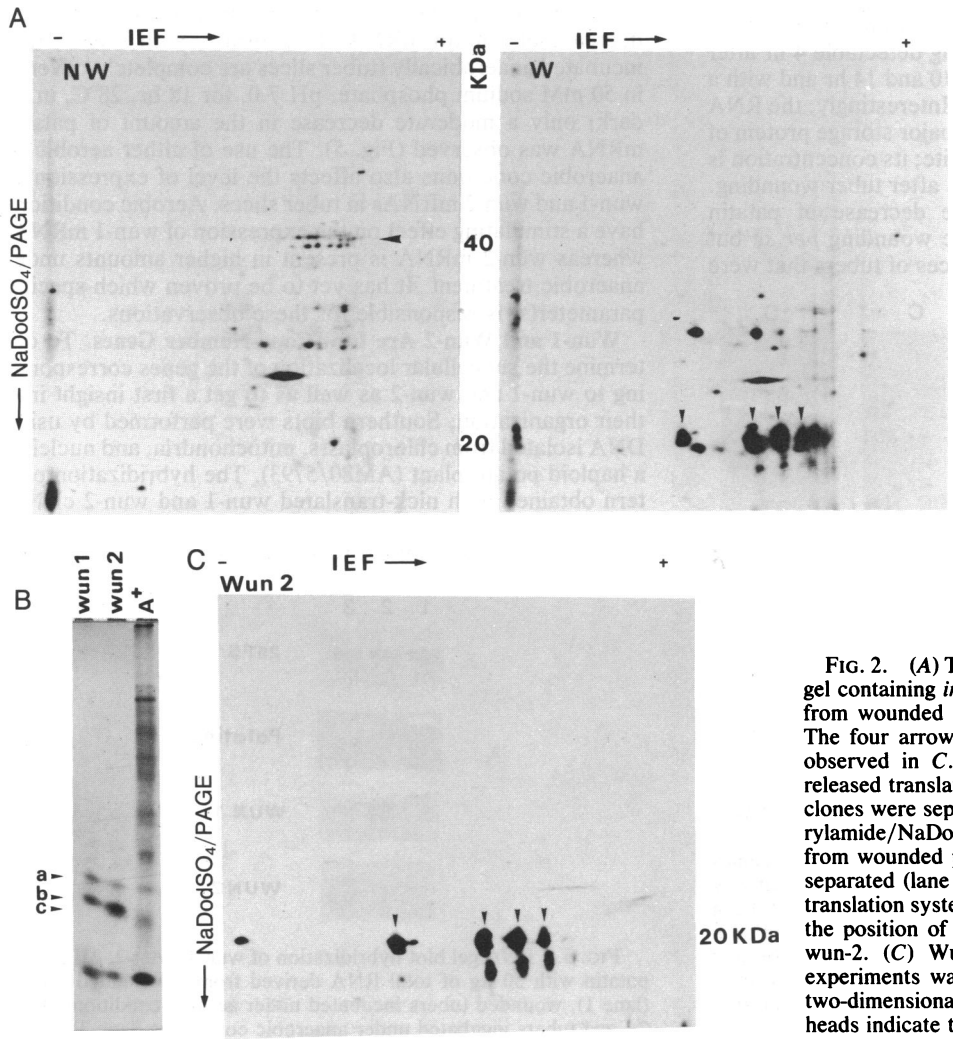

FIG. 2. (*A*) Two-dimensional 15% (wt/vol) acrylamide gel containing *in vitro* translated poly$(A)^+$ RNA isolated from wounded (W) and unwounded (NW) tuber tissue. The four arrowheads indicate the same protein pattern observed in *C*. IEF, isoelectric focusing. (*B*) Hybrid-released translation products of wun-1 and wun-2 cDNA clones were separated on a one-dimensional 12% polyacrylamide/$NaDodSO_4$ gel. As a control, poly$(A)^+$ RNA from wounded potato tubers was translated *in vitro* and separated (lane A^+). An endogenous protein due to the translation system used is indicated by a; b and c indicate the position of the polypeptides encoded by wun-1 and wun-2. (*C*) Wun-2 RNA isolated by hybrid released experiments was translated *in vitro* and separated on a two-dimensional 15% acrylamide gel. The four arrowheads indicate the specific pattern of the protein.

wounded and nonwounded tubers using nick-translated wun-1 and wun-2 as probes. Both cDNA clones hybridized only to RNA of wound-induced tubers (Fig. 3). The size of the RNA bands corresponding to these cDNA clones was 800 base pairs (bp) for wun-1 and 750 bp for wun-2.

Both cDNA clones encode a protein as indicated by the formation of a polypeptide on a one-dimensional acrylamide $NaDodSO_4$ gel in an *in vitro* translation system using hybrid-selected mRNAs (Fig. 2*B*). The wun-2 protein can also be seen as prominent 20-kDa spots on a two-dimensional gel (Fig. 2*C*). The question whether the four spots indicated by arrowheads are due to isoproteins of a small wun-2 gene family or to posttranslational modifications of one protein type in the *in vitro* translation system (18) is still open. The observation that there are also spots with slightly lower molecular weights just below the other four spots might be explained by a degradation event during the isoelectric focusing. A degradation during the $NaDodSO_4$ treatment can be ruled out, because no differences in the molecular weight are detectable on a one-dimensional $NaDodSO_4$ gel (Fig. 2*B*). Since identical spots can be observed after *in vitro* translation of total poly(A)$^+$ RNA (Fig. 2*A*; see four arrowheads), the presumed degradation must be specific for wun-2-encoded proteins.

To analyze the time course of the expression of wun-1, wun-2, and patatin genes, kinetics were established by using RNA from tubers isolated at various intervals after wounding.

Fig. 4 shows that wun-1 homologous RNA (800 bp) can already be detected 30 min after wounding. The highest amount of RNA is reached after 10 hr and remains constant for the next 14 hr. The expression of wun-2-homologous RNA (750 bp) is slightly delayed being detectable 4 hr after wounding with a maximum between 10 and 14 hr and with a slight decrease 24 hr after wounding. Interestingly, the RNA corresponding to the patatin gene, a major storage protein of potato tubers, behaves just the opposite; its concentration is dramatically decreased within 30 min after tuber wounding. Further experiments show that the decrease of patatin mRNA is not only dependent on the wounding *per se* but also on the incubation conditions. Slices of tubers that were

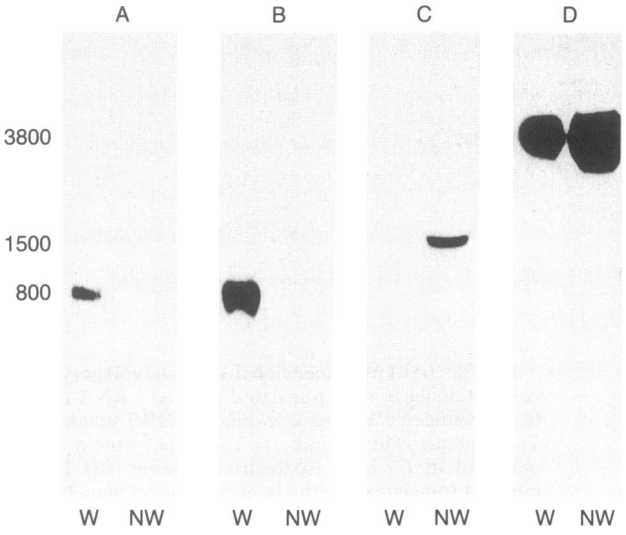

FIG. 3. RNA gel blot analysis of three wound-dependent clones [wun-1 (*A*), wun-2 (*B*), and patatin (*C*)] and the constitutively expressed clone 25F5 (*D*). Total RNAs (50 μg) isolated from wounded (W) and not wounded (NW) tubers were separated on a 1.5% denaturing formaldehyde gel, blotted onto nitrocellulose, and hybridized to ^{32}P-labeled nick-translated cDNA inserts of wun-1, wun-2, patatin, and 25F5. Sizes of the specific RNAs are indicated in nucleotides.

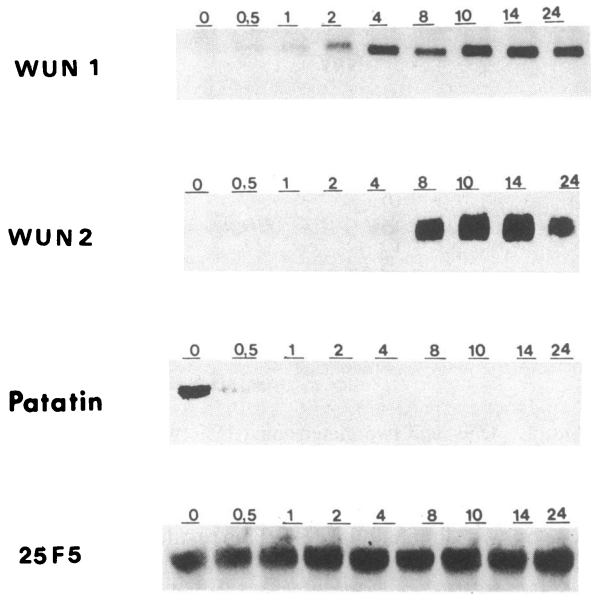

FIG. 4. RNA gel blot hybridization of wun-1, wun-2, and patatin cDNAs with total RNA isolated at various times (in hr as indicated by the lane labels) after the wounding of potato tubers. Total RNAs (50 μg) were separated by denaturing formaldehyde 1.5% gel electrophoresis and after transfer onto nitrocellulose hybridized to ^{32}P-labeled nick-translated wun-1, wun-2, and patatin cDNA inserts.

incubated under aerobic conditions (dipped once into the 50 mM sodium phosphate, pH 7.0, and then incubated dry for 18 hr at 28°C in the dark) showed the above mentioned decrease of patatin mRNA. In contrast, with tuber slices incubated anaerobically (tuber slices are completely covered in 50 mM sodium phosphate, pH 7.0, for 18 hr, 28°C, in the dark) only a moderate decrease in the amount of patatin mRNA was observed (Fig. 5). The use of either aerobic or anaerobic conditions also affects the level of expression of wun-1 and wun-2 mRNAs in tuber slices. Aerobic conditions have a stimulating effect on the expression of wun-1 mRNA, whereas wun-2 mRNA is present in higher amounts under anaerobic treatment. It has yet to be proven which specific parameter(s) is responsible for these observations.

Wun-1 and Wun-2 Are Low-Copy-Number Genes. To determine the subcellular localization of the genes corresponding to wun-1 and wun-2 as well as to get a first insight into their organization, Southern blots were performed by using DNA isolated from chloroplasts, mitochondria, and nuclei of a haploid potato plant (AM80/5793). The hybridization pattern obtained with nick-translated wun-1 and wun-2 cDNA probes showed that homology is detectable only with plant nuclear DNA (data not shown). The number of hybridizing

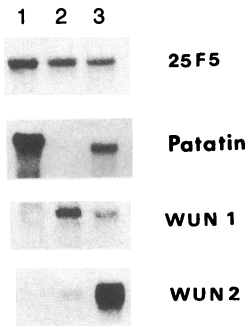

FIG. 5. RNA gel blot hybridization of wun-1, wun-2, 25F5, and patatin with 50 μg of total RNA derived from unwounded tubers (lane 1), wounded tubers incubated under aerobic conditions (lane 2), and tubers incubated under anaerobic conditions (lane 3).

bands is low (one main band) (Fig. 6) indicating that wun-1 and wun-2 are most likely coded for by small gene families in contrast to the multigene family encoding patatin (9).

Wound-Dependent Expression of wun-1, wun-2, and Patatin Is Controlled at the Level of Transcription. The expression of nuclear genes in response to environmental or developmental signals can be controlled at either the transcriptional or posttranscriptional level (19). To discriminate between these possibilities, run-off transcription experiments were performed using nuclei isolated from wounded and nonwounded Granola tubers. The hybridization of RNA synthesized in isolated nuclei with various cDNA clones immobilized on nitrocellulose filters is shown in Fig. 7. Both coding and noncoding strands of the cDNA clones were hybridized. No hybridization signal was detected against the noncoding strand of any of the offered clones confirming the fidelity of the transcription. Wun-1 and wun-2 show a clear hybridization only with RNA synthesized in nuclei isolated from wounded tubers; there is no signal detectable using RNA from nuclei of nonwounded tubers. The coding strand of patatin on the other hand hybridizes specifically to RNA from nuclei of nonwounded tubers.

RNA Gel Blot Analysis Demonstrates Organ- and Wound-Specific Expression of Patatin mRNA. RNA was isolated from stem, leaf, and root of a tetraploid potato plant (Granola) to analyze whether the wound-induced suppression of patatin expression is restricted to potato tubers or occurs also in other organs. Fig. 8 shows the hybridization of a patatin cDNA probe to various RNAs. By using unwounded tubers, patatin mRNA is detectable in stems and roots but not in leaves. No patatin mRNA was observed in leaves, roots, or stems after wounding. Therefore, wounding leads to a reduction of patatin mRNA in all organs in which it is normally expressed.

Considerable amounts of wun-1 and wun-2 homologous RNA were detected in stems, whereas roots and leaves contained less wun-1 and wun-2 RNA (data not shown). No consistent results were obtained concerning wound inducibility of wun-1 and wun-2 in these organs, which in some experiments increase after wounding.

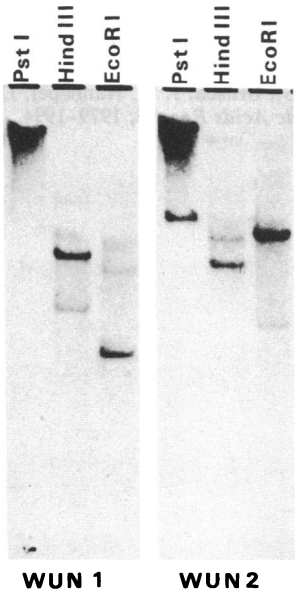

FIG. 6. Southern blot analysis of wun-1 and wun-2. Nuclear DNA of a haploid potato genotype AM80/5793 (8 μg) was digested to completion with *Pst* I, with *Eco*RI, or with *Hin*dIII, separated on a 0.8% agarose gel, and blotted onto nitrocellulose. These filters were hybridized with ^{32}P-labeled nick-translated wun-1 and wun-2 cDNA inserts.

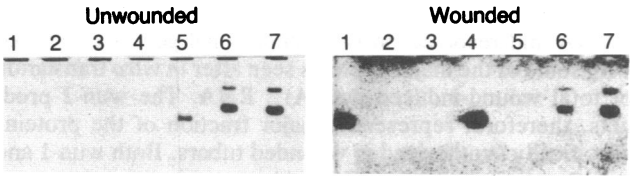

FIG. 7. Comparison of run-off transcripts of wound-dependent clones in nuclei isolated from tubers after wounding or from unwounded tubers. A total of 500 ng of the coding strand and the noncoding strand of wun-1 and wun-2, the coding strand of patatin, and DNA of 25F5 were electrophoresed on a 0.8% agarose gel and subsequently blotted on nitrocellulose filters. These were hybridized with total RNA pulse-labeled in isolated nuclei from tubers after wounding and without wounding. Lane 6 and 7 contain 25F5 DNA, which shows homology to rRNA was used as an internal control. Lanes: 1, wun-1 coding strand; 2, wun-1 noncoding strand; 3, wun-2 noncoding strand; 4, wun-2 coding strand; 5, patatin coding strand; 6 and 7, 25F5 DNA.

Wound Is the Main Determinant Inducing the Expression of Genes Corresponding to wun-1 and wun-2. To prove that actually wounding is the primary cause for the induction of wun-1 and wun-2 gene expression in sliced potato tubers, potato plants were incubated at various temperatures (4, 18, 28, or 37°C) for 18 hr without wounding. No expression of wun-1 and wun-2 was detected at the RNA level in tubers (data not shown). In a similar way changing the buffer or the light conditions during incubation did not result in induction of wun-1 and wun-2 (data not shown) indicating wounding to be the primary cause for the induction of expression of both clones.

DISCUSSION

This study analyzed the effect of wounding on gene expression in potato tubers. Wounding leads within hours to the activation of transcription of some genes (wun-1 and wun-2) and to the suppression of transcription of others such as the patatin genes, whereas transcription of still other genes coding, e.g., 25S rRNA, is not affected by wounding. These dramatic changes become evident by analyzing *in vitro* translation products of mRNA before and after wounding and are not readily detected by examining steady-state proteins (compare Figs. 1 and 2 *A* and *B*).

By differential screening of a cDNA library derived from wound-induced mRNA, two wound-specific cDNA clones, wun-1 and wun-2, were isolated.

Hybrid-selected translation of wun-1 and wun-2 mRNA resulted in the synthesis of 18- to 20-kDa proteins. The

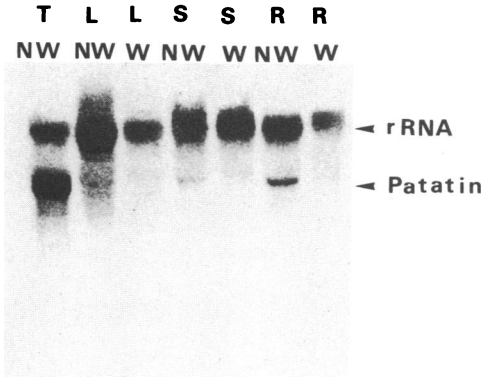

FIG. 8. RNA gel blot hybridization of ^{32}P-labeled nick-translated patatin cDNA insert with 50 μg of total RNA derived from tubers (lane T), leaves (lanes L), stems (lanes S), and roots (lanes R) from wounded (W) and not wounded (NW) tubers.

specific protein pattern obtained by translation of wun-2 mRNA and resolved on two-dimensional gels is correlated with some of the major proteins seen after *in vitro* translation of total wound-induced poly(A)$^+$ RNA. The wun-2 products, therefore, represent a major fraction of the proteins specifically synthesized in wounded tubers. Both wun-1 and wun-2 correspond to single- or low-copy genes that upon induction give rise to mRNAs of 800 and 750 nucleotides, respectively. Run-off experiments showed that induction results from initiation of transcription. Wun-1 was very rapidly induced, since wun-1-specific mRNA was already detectable 30 min after wounding of tubers. This rapid induction is similar to that of the proteinase inhibitor II in potato leaves (H. Pena-Cortes, personal communication). The induction of transcription of wun-2 is considerably slower, since mRNA cannot be detected prior to 4 hr after wounding.

Wounding does not only result in gene activation but most likely also in a very rapid shutoff of transcription of certain genes. This is illustrated by the patatin gene family. Within 30 min after wounding of tubers most of the steady-state mRNA for patatin has disappeared, and consequently *in vitro* translation of mRNA from wounded tubers does not yield patatin proteins. Run-off experiments demonstrate that wounding rapidly leads to a suppression of patatin transcription initiation. Presumably because of the stability of the patatin protein, this shutoff of transcription does not lead to a notable decrease in the steady-state amount of patatin proteins in wounded tubers. Apparently the biosynthetic capacity of tubers is redeployed after wounding in favor of defense and repair mechanisms. This shift in protein synthesis appears to be regulated at the transcriptional level.

The observation that patatin is not only present in unwounded roots and tubers but also, in much lower levels, in unwounded stems of intact potato plants, to our knowledge, has not been described (9, 20). The lack of detection of patatin mRNA (9) might be due to the fact that the amount of patatin hybridizing RNA in stems is very low. Another possible explanation would be that patatin mRNA is formed in stems during certain developmental stages only. In this respect it is important to note that RNA hybridizing to patatin cDNA was found in stems of potato plants that had not yet tuberized. More extensive experiments are necessary to determine whether there is a correlation between tuber formation and the appearance of patatin mRNA in stems. The reduction of patatin mRNA in all three organs after wounding leads to the conclusion that the mechanism responsible for transcription shutoff is not organ specific.

In conclusion the three potato clones described here form a system that can be used to study the effect of wounding on gene expression in tubers.

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