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Expression of a Gene Encoding a Glycine-Rich Protein in Petunia

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We have investigated the expression of a gene that codes for a glycine-rich structural protein (GRP1) in petunia. This gene is expressed as a single polyadenylated RNA of approximately 1,600 bases which was found to be present in leaves, stems, and flowers of petunia but not in roots. In the organs in which GRP1-specific mRNA was expressed, its steady-state levels were highest in stems and leaves and lowest in flowers. This analysis also revealed that the pattern of organ-specific expression for several of the GRP1-related genes was distinctly different. In addition, it was found that the levels of GRP1 RNA were significantly higher in young leaves and stems than in old, implying developmental regulation of the gene. GRP1-specific RNA in both old and young tissue that had been wounded was found to be increased at least 25-fold over that in young unwounded tissue. Increased levels of GRP1 mRNA were seen within 5 min after wounding, with substantial increases apparent by 30 min. Maximal levels of accumulation of GRP1 transcripts occurred 90 min after wounding. The enhancement of GRP1 mRNA levels by wounding appears to be one of the earliest events of the plant wound response and is distinct from that which we observed for the *PAL* gene in petunia. Using S1 analysis and RNA primer extension, we demonstrated that the same transcriptional start site was used by the GRP1 gene in all organs and in wounded and unwounded tissue. The potential significance of these data with regard to wound signal transduction is discussed.

The major components of the plant cell wall are cellulose, hemicellulose, lignin, pectic polysaccharide, and structural protein. The last component is presumed to provide a scaffolding for the architecture of the cell wall and in addition may provide the elasticity to the cell wall necessary for cell growth by extension (18). To data, only one cell wall structural protein of plants has been isolated and well characterized. This protein, extensin or hydroxyproline-rich glycoprotein (HRGP), was first isolated based on the fact that cell walls of many plant species contain high amounts of the unusual imino acid hydroxyproline (23). HRGPs have been isolated from many species, including tobacco, tomato, carrot, and soybean (4, 5, 7, 14, 25, 26) and it is now generally accepted that extensins or HRGPs are ubiquitous cell wall structural proteins in dicotyledonous plants. However, extensins or HRGPs cannot be the only cell wall structural protein of plants, as some plant species and organs contain very little hydroxyproline in their cell walls but instead contain large amounts of glycine (29). This indicates that some plant cell walls may contain primarily glycine-rich rather than hydroxyproline-rich structural proteins.

We have recently reported the isolation and sequencing of an expressed gene from petunia encoding a glycine-rich protein which we have named glycine-rich protein 1 (GRP1) (6). It is likely that this gene functions as a cell wall structural or cell wall-associated protein in petunia for the following reasons. (i) The predicted amino acid composition of the protein is 67% glycine, making it unlikely that it could code for anything other than a structural protein. (ii) The carboxy-terminal 317 amino acids of the protein are capable of forming an eight-stranded β-pleated sheet. (iii) The first 27 amino-terminal residues compose an optimal signal sequence for transport out of the cytoplasm (4). Cassab and Varner have isolated from pumpkin seed coats a glycine-rich protein fraction which has the same density as is predicted for the GRP1 gene product (1.58 g/ml) (6). Their work provides support for the hypothesis that GRP1 is a structural cell wall protein and suggests that GRPs (in varying amounts) may be found in many plant species.

In this study we investigated the steady-state levels of GRP1-specific mRNA to determine whether the pattern of expression of this gene is consistent with its predicted role as a cell wall or cell wall-associated protein. A gene with this function would be expected to be developmentally regulated and induced by wounding.

MATERIALS AND METHODS

Constructions. Figure 1 is a physical map of the GRP1 gene and the three GRP1 subclones used in this study. p7D3-500 and p7Sph-Pst were subcloned into pTZ19U (U.S. Biochemical Corp.) from our p7RI-Pst clone, which has been described (6). The orientation of both inserts in their pTZ19U vectors is such that antisense strand RNA would be synthesized from the T7 RNA polymerase promoter. The cloning of p7D3-1200 has been described previously. pPAL5 is a cDNA clone of phenylalanine-ammonia lyase (PAL) isolated from French bean (*Phaseolus vulgaris* L.) (9) generously provided by C. Lamb. The 1,063-base-pair (bp) *Rsa*I fragment of pPAL5 used in this study contains only coding sequence.

RNA isolation and plant material. Plant material used in this study was from the Mitchell strain of *Petunia hybrida*. Tissue was harvested, frozen in liquid nitrogen, and stored at −70°C until use. RNA was isolated by a modification of a DNA extraction procedure described previously (24). In brief, tissue was first ground in a coffee grinder. The powdered plant tissue was taken up in 8 ml of grinding buffer (24) per g of starting tissue. This mixture was then processed for 1 min in a Polytron at a setting of 9. An equal volume of a 3:1 mixture of phenol and chloroform containing 5% isoamyl alcohol was added. This mixture was then shaken for 45 to 60 min at 250 rpm. The phases were separated by low-speed centrifugation, and the phenol-chloroform-isoamyl alcohol extraction was repeated twice. After ethanol precipitation, the nucleic acid pellet was dissolved in 1 ml of 10 mM Tris

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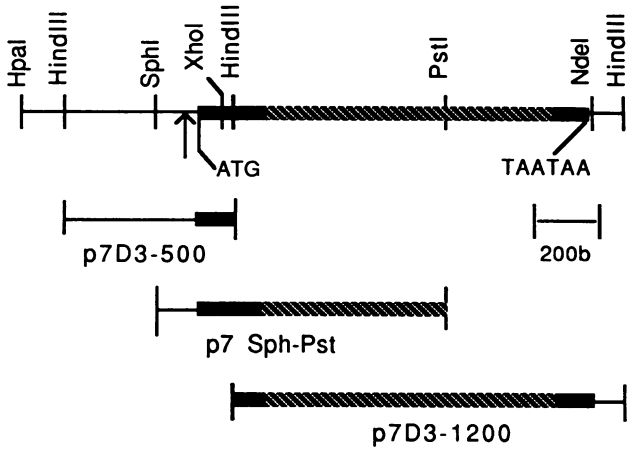

FIG. 1. (Top) Restriction map of the GRP1 gene and flanking sequences. (Bottom) Three subclones of the GRP1 region used in this study. The heavy bar indicates the 1,152-nucleotide coding region of the GRP1 gene. The striped area within the bar indicates the position of the two families of seven structural repeats of the GRP1 gene product. The arrow indicates the transcriptional start of the GRP1 gene. ATG, Presumptive translation start signal; TAATAA, presumptive translation stop signals.

(pH 7.4)–1 mM EDTA per 10 g of starting weight. The solution was made 2 M LiCl and placed overnight at 0°C. Single-stranded RNA was recovered by low-speed centrifugation. This pellet was washed twice with cold 2 M LiCl, suspended in 10 mM Tris (pH 7.4)–150 mM NaCl–1 mM EDTA, ethanol precipitated, and stored at −70°C in ethanol.

Leaf tissue in this study includes both petiole and blade, and unless otherwise stated (see below) only the first four to six apical expanded leaves were used. Flower tissue included the corolla, stigma, style, and stamens. Root tissue was prepared either from plants grown in soil with roots washed free of dirt or from plants first grown in soil and then transferred and grown in water for 3 weeks. Young tissue (wounded or unwounded) is defined (for Fig. 3) as a mixture of leaves and stem of the top 6 cm of an approximately 30-cm stem, while old tissue (wounded or unwounded) is defined as a mixture of leaves and stem of the bottom 6 cm of an approximately 30-cm stem. Wounding of tissue was accomplished by one of the two following methods. Stems and leaves were cut sterilely with scissors into segments of 1 to 2 by 5 to 6 mm (Fig. 3, lanes OW and YW; Fig. 4B and D), or leaves were chopped with an onion chopper (Gemco Ware, Inc., Freeport, N.Y.). Leaves or leaves and stems that had been wounded were immediately placed in sterile plastic petri dishes containing 8 to 15 sheets of sterile Whatman 3MM paper which had been saturated with sterile water. The petri dish was sealed with parafilm and incubated on the bench top at room temperature approximately 20 cm beneath a 22-W white circular fluorescent light for various periods of time. After incubation, the tissue was frozen in liquid nitrogen and stored at −70°C until used. The time points indicated in Fig. 4 do not include the 2- to 3-minute tissue preparation time.

Northern analysis, S1 analysis, and primer extension. For Northern (RNA blot) analysis, RNA was denatured, electrophoresed, and blotted to nitrocellulose as described previously (6). Hybridization to nick-translated DNA (19) was performed in the following buffers: (Fig. 2A) 50% formamide, 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 10× Denhardt, 50 mM PO_4, pH 7.0, 0.2% sodium dodecyl sulfate (SDS), tRNA (250 μg/ml) at 50°C, to ^{32}P-labeled p7D3-1200; (Fig. 3) same hybridization buffer as for Fig. 2A, but hybridization was performed at 65°C to ^{32}P-labeled p7D3-1200; (Fig. 2B, lanes 1 and 2, and Fig. 4A and B) 50% formamide, 2× SSC, 10× Denhardt, 50 mM PO_4, pH 7.0, 0.2% SDS, tRNA (250 μg/ml) at 56°C to ^{32}P-labeled p7D3-1200; (Fig. 2B, lanes 3 and 4 and Fig. 4C and D) 6× SSC, 10× Denhardt, 50 mM $NaPO_4$, pH 7.0, 0.5% SDS tRNA (250 μg/ml) at 56°C to the ^{32}P-labeled 1,063-bp *Rsa*I fragment of pPAL5 (9). Blots which were hybridized in a solution containing formamide were washed twice for 15 min each in 2× SSC–0.2% SDS and twice for 15 min each in 0.1× SSC–0.2% SDS at 65°C. Blots which were hybridized in aqueous solution were washed three times for 15 min in 6× SSC–0.5% SDS at 56°C.

S1 analysis was first performed to determine the approximate 5′ and 3′ ends of the GRP1 mRNA and also to determine whether introns were present in the mRNA. The method used in the later analysis is diagramatically presented in Fig. 5. In brief, 10 μg of polyadenylated (PA^+) RNA isolated from wounded stem and leaf tissue were hybridized (10) at 55°C to 25 ng of unlabeled *Sph*I-*Pst*I-cut p7Sph-Pst or 25 ng of unlabeled *Hin*dIII-cut p7D3-1200 and digested with 1,000 U of S1 per ml in a final volume of 200 μl (10). The RNA-DNA hybrids were ethanol precipitated, denatured, electrophoresed, and transferred to nitrocellulose as described above. The blot was hybridized to the inserts of nick-translated p7D3-1200 and p7Sph-Pst and washed as indicated for Fig. 4A and B above.

For the specific 5′-end mapping of the GRP1 mRNA, the insert of p7D3-500 was ^{32}P-labeled at the 5′ positions with calf alkaline phosphatase (Boehringer Mannheim) and polynucleotide kinase (Bethesda Research Laboratories), digested with *Sph*I, and the 246-bp *Sph*I-*Hin*dIII fragment (comprising nucleotides 456 to 656 of Fig. 2 of reference 6) was isolated from a 5% acrylamide gel. This fragment was then hybridized for 18 h at 42°C to 5 to 20 μg of PA^+ RNA (10). The RNA-DNA hybrids were digested with 750 U of S1 per ml in 200 μl as described (10). The resulting DNA fragment was then analyzed on a 7.5% acrylamide–7 M urea gel and identified by autoradiography. 3′-end mapping was performed in a similar manner. The insert of p7D3-1200 was recloned into the *Hin*dIII site of pTZ19U. This plasmid was digested with *Nde*I and labeled at the 3′ position with Klenow and digested with *Hin*dIII and the 142-bp *Nde*I-*Hin*dIII fragment was isolated from a 5% acrylamide gel. Hybridization and S1 digestion were performed as above except that the hybridization temperature was 33 or 37°C.

For primer extension analysis, p7D3-500 was cut with *Hin*dIII, and the 5′ ends were labeled as above. The labeled fragments were digested with *Xho*I, and the 46-bp *Xho*I-*Hin*dIII fragment was isolated from a 5% acrylamide gel. PA^+ RNA (5 to 20 μg) was hybridized to the denatured fragment at 30°C for 18 h (28). THe hybrids were recovered by ethanol precipitation, suspended in 25 μl of primer extension buffer (60 mM Tris, pH 8.0, 0.5 mM EDTA, 10 mM dithiothreitol, 8.5 mM $MgCl_2$, 50 mM NaCl, 0.5 mM each dATP, dCTP, dGTP, and TTP, and 20 U of reverse transcriptase [Promega Biotec]), and incubated at 42°C for 1 h. Primer extension products were analyzed on 7.5% acrylamide–7 M urea gels by autoradiography.

RESULTS

Organ-specific expression. To assay the organ specificity of GRP1 expression, PA^+ RNA was isolated from petunia

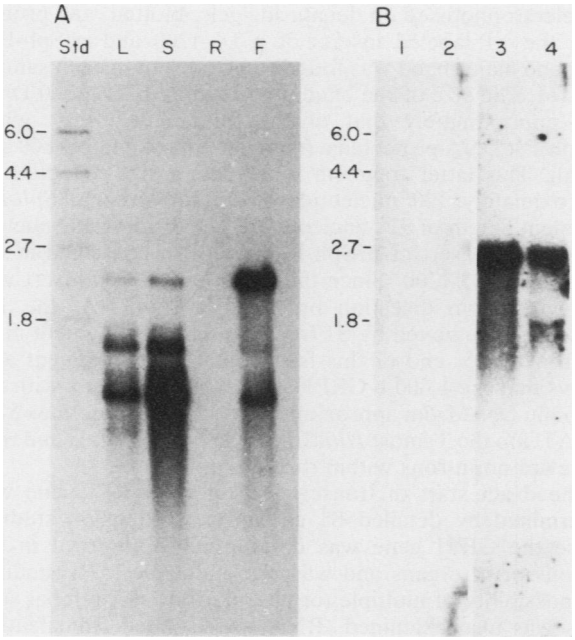

FIG. 2. Organ-specific expression of GRP1-related transcripts. (A) PA^+ RNA (10 μg) isolated from leaves (L), stems (S), roots (R), or flowers (F) was electrophoresed, blotted, and hybridized to ^{32}P-labeled p7D3-1200. (B) RNA (50 μg, lanes 1 and 3; 25 μg, lanes 2 and 4) isolated from roots grown in nonsterile water was electrophoresed, blotted, and hybridized to ^{32}P-labeled p7D3-1200 (lanes 1 and 2) or the 1,063-bp *Rsa*I fragment of pPAL5 (lanes 3 and 4). Numbers to the left of each blot are the sizes (in kilobase) of DNA standards (Std) run in parallel.

leaves, stems, roots, and flowers and examined by Northern analysis. Figure 2A shows the presence of three strongly hybridizing RNA species in leaves, stems, and flowers (lanes L, S and F). These three transcripts represent the previously reported GRP1-related mRNAs of 2,200, 1,600 and 1,200 bases (b) (6). Several other weakly hybridizing RNAs were also discernable, including the previously reported 1,700-b GRP1-related transcript (6) (see below). The expression of the 2,200-, 1,700-, 1,600-, and 1,200-b GRP1-related transcripts was not detectable in root tissue (lane R). Furthermore, these four RNAs seemed to be more highly expressed in stems than in leaves. A direct comparison of the steady-state levels of GRP1-related transcripts in these two organs could be made because stem RNA was prepared only from portions of the stem from which leaves had been harvested for the preparation of leaf RNA. The expression of the four RNAs in flowers was shown to be highly variable, with the 2,200-b RNA appearing as a high-abundance mRNA in this organ.

Root tissue used to prepare RNA in the above experiment was isolated from plants grown in soil; the roots were washed free of dirt. It is possible that this method of preparation could cause the loss of fragile, growing root tips. Because the GRP1 gene was subsequently found to be expressed at high levels in young tissue and barely detectable in old tissue (see below), it is possible that in the above experiment we inadvertently excluded from analysis the type of root tissue in which the GRP1 gene is expressed. This experiment was therefore repeated with RNA isolated from root tissue of young plants, grown first in soil and then for 3 weeks in nonsterile water. The quantity of root tissue approximately doubled during the 3-week growth period. This root-specific RNA, when examined by Northern analysis (lanes 1 and 2, Fig. 2B), also contained undetectable levels of GRP1-related transcripts. As a positive control, the ^{32}P-labeled 1,063-bp *Rsa*I fragment of pPAL5, partially encoding the PAL gene from bean (9), was then hybridized to this same blot. The PAL gene is known to be expressed in roots, and its expression is enhanced by wounding and by elicitor (2). A single RNA species of approximately 2,600 b was readily apparent (Fig. 2B, lanes 3 and 4). We could thus easily detect, with a heterologous probe, the transcript encoding the PAL gene, but were unable to detect any GRP1-related transcripts in roots. This indicates that the GRP1 gene is either not expressed in roots or is expressed at such low levels as to be undetectable by the methods used in these experiments.

GRP1 gene codes for a single transcript. Comparison of the results in Fig. 2A with those previously reported from GRP1 expression studies (6) shows a quantitative difference in the relative amounts of the four GRP1-related RNAs. Most significantly, the amount of the 1,600-b RNA was much greater than that of the other three RNAs, while the amount of the 1,700-b RNA was much less than previously reported. As the blot in Fig. 2A was hybridized in formamide at a more stringent temperature than previously (50 versus 42°C), this suggested that the 1,600-b RNA might be the actual transcript of the GRP1 gene. This drastic decrease in hybridization to the 1,700-b RNA confirmed our previous results that the 1,700-b RNA was the least related of the four RNAs to the GRP1 gene and was most likely derived from a distant but related gene family member. To determine whether the 1,600-b RNA was the sole GRP1-specific mRNA, PA^+ RNA was isolated from young leaf and stem tissue and blotted to nitrocellulose, and the imprint was hybridized to ^{32}P-labeled p7D3-1200 in formamide at 65°C. Under such stringent conditions, only the 1,600-b RNA hybridized well to GRP1 DNA (Fig. 3 lane Y). These extreme hybridization conditions are apparently required due to the high G+C composition of GRP1 and the GRP1-related genes.

Developmental expression and wound activation. Our working hypothesis has been that the GRP1 gene codes for a cell wall or cell wall-associated protein. This hypothesis predicts that the GRP1 gene would be developmentally regulated. It also predicts that when the cell wall is damaged, as by wounding, the level of GRP1-specific mRNA should increase to effect repair of the cell wall. To test this hypothesis, PA^+ RNA was isolated from young and old tissue before and 18 h after wounding (see above). The levels of GRP1 mRNA were much greater in young than in old tissue, and the levels of mRNA in either young or old wounded tissue was enhanced approximately 25-fold above the level in young unwounded tissue (Fig. 3). Rehybridization of this blot at a lower stringency showed that the levels of the 1,200-b GRP1-related RNA did not vary between young and old or wounded tissue (data not shown), suggesting that although this mRNA is very closely related to the GRP1 gene on the nucleotide level, its gene product may function in a capacity unrelated to that of the GRP1 gene product. Expression of the 2,200-b RNA appeared to be developmentally regulated in a manner similiar to the GRP1 gene, but its activation by wounding appeared to be quantitatively different. Interestingly, the 1,700-b RNA, which was shown previously to be the least related of the four RNAs (6), exhibited the same pattern of expression as the GRP1 transcript. Thus, the 1,700-b and 2,200-b RNAs are most likely coded for by different members of the GRP gene family.

The kinetics of the accumulation of the GRP1 transcript

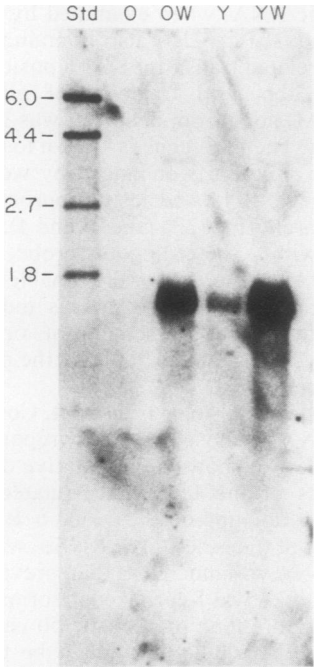

FIG. 3. Autoradiograph of RNA blot hybridized to ^{32}P-labeled p7D3-1200. Lanes: 0, 10 μg of PA$^+$ RNA isolated from old leaves and stems (see Materials and Methods); OW, 10 μg of PA$^+$ RNA isolated from old leaves and stems 18 h after wounding; Y, 10 μg PA$^+$ RNA isolated from young leaves and stems; YW, 10 μg of PA$^+$ RNA isolated from young leaves and stems 18 h after wounding; Std, DNA molecular size standards. The sizes of the standards are indicated to the left (in kilobases).

after wounding was determined by RNA blot hybridization. RNA for this analysis was isolated from leaf tissue at various time points after wounding. Accumulation of transcripts was seen to occur within 5 min after wounding, with maximal accumulation occurring by 90 min (Fig. 4A). The level of GRP1 mRNA slowly declined after 90 min. However, 24 h after wounding, the level of GRP1 transcripts was still 5- to 10-fold greater than that present in unwounded tissue (Fig. 4B). The relative contribution of changes in mRNA stability or transcriptional rate in the accumulation of the GRP1 transcript after wounding has not been determined.

To determine the temporal relationship of the accumulation of GRP1 transcripts to other genes activated by the wound response, the ^{32}P-labeled 1,063-bp *Rsa*I fragment of pPAL5 was hybridized to the same blots shown in Fig. 4A and B. In petunia, an increase in the level of PAL gene transcripts was detected within 5 min after wounding (Fig. 4C), with maximum steady-state levels reached by 90 min (Fig. 4D). Thus, the early kinetics of the accumulation of PAL transcripts directly paralleled that of GRP1. However, unlike GRP1, the maximal level of expression of PAL mRNA was maintained even after 24 h (Fig. 4D). The same blots were also hybridized to the insert of a clone which contained the sequences encoding soybean 18S rRNA (8). No significant differences in the amounts of 18S rRNA were seen between samples (data not shown).

Transcriptional start of the GRP1 gene. To define the endpoints of the GRP1 mRNA, PA$^+$ RNA isolated from wounded stem and leaf tissue was subjected to S1 analysis (Fig. 5). When this RNA was hybridized to either *Sph-Pst*-cut p7Sph-Pst or *Hin*dIII-cut p7D3-1200 DNA, treated with S1, electrophoresed on denaturing gels, blotted, and probed with the ^{32}P-labeled inserts of p7D3-1200 and p7Sph-Pst, only one major band was found to be present in each sample (Fig. 6). The size of the band protected by p7D3-1200 DNA was approximately that of the full-length insert, while p7Sph-Pst DNA protected a fragment approximately 800 b in length. This latter fragment represents a size reduction of approximately 120 nucleotides from the original *Sph-Pst* fragment length of 918 nucleotides. The 3′-most 662 nucleotides of the insert of p7Sph-Pst were also contained in the insert of p7D3-1200. Since the entire p7D3-1200 insert was protected from digestion by the GRP1 mRNA, the 120 nucleotides removed by S1 from the *Sph-Pst* fragment must be from the 5′ end of this fragment. This experiment also shows that the 1,600-b GRP1 RNA was contiguous with the genomic DNA from approximately 30 to 50 nucleotides 5′ of the ATG to the 3′-most *Hin*dIII site shown in Fig. 1, and thus there are no introns within this region.

The exact start of transcription of the GRP1 gene was determined by detailed S1 and primer extension studies. Since the GRP1 gene was differentially expressed in the various plant organs and was also inducible by wounding, the possibility of multiple (or alternate) transcriptional start sites was also examined. RNA was isolated from leaves, stems, flowers, and wounded tissue and analyzed. S1 analysis was performed on RNA hybridized to the 246-bp *Sph*I-*Hin*dIII fragment labeled at the *Hin*dIII site. Primer extension studies were performed with the 46-bp *Xho*I-*Hin*dIII fragment labeled at the *Hin*dIII site as a primer. A fragment 134 b in length was obtained by both primer extension and S1 protection of RNA from all three organs and also wounded tissue (Fig. 7). This indicates that tran-

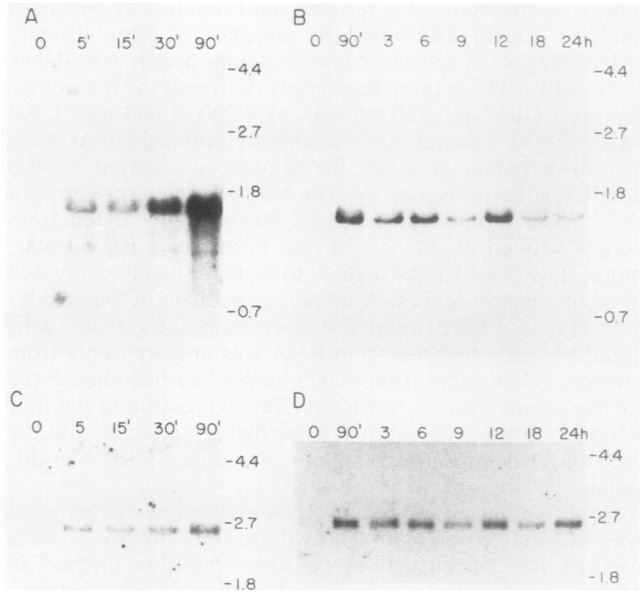

FIG. 4. Kinetics of GRP1- and PAL-specific mRNA accumulation in petunia after wounding. Total RNA was isolated from petunia leaves after wounding at the times indicated (minutes or hours). RNA (25 μg) from each time point was electrophoresed, blotted, and hybridized to ^{32}P-labeled p7D3-1200 (A and B) or the ^{32}P-labeled 1,063-bp *Rsa*I fragment of pPAL5 (C and D). Numbers to the left indicate the sizes of DNA standards run in parallel (in kilobases). Hybridization conditions for panels A and B were as indicated in the Materials and Methods section and are equivalent to the gene-specific conditions used in Fig. 3.

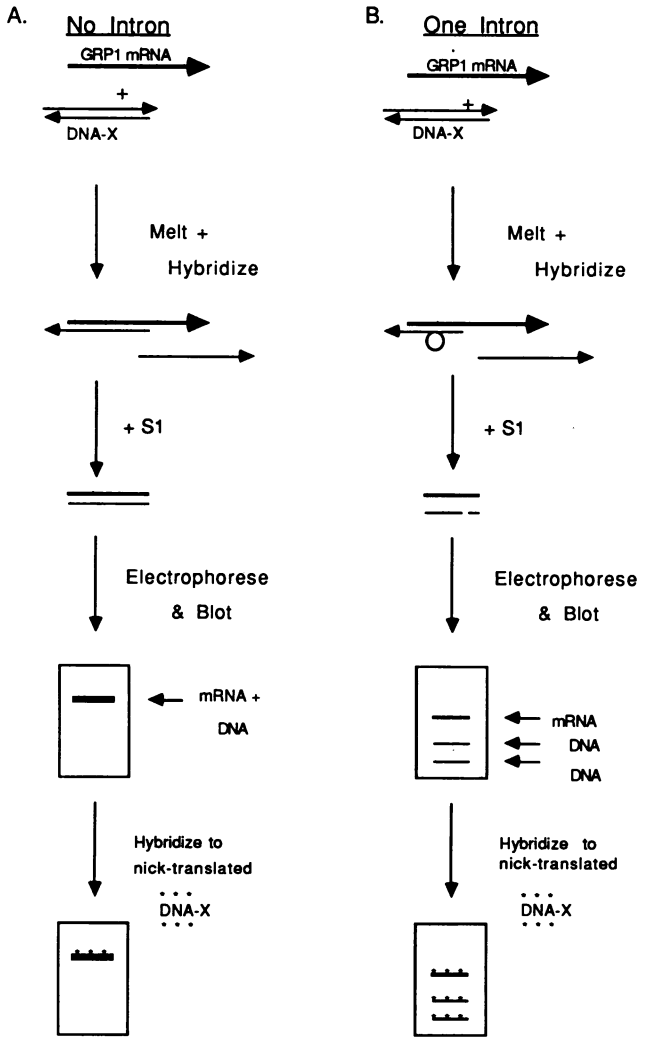

FIG. 5. Strategy for determining the structure of the GRP1 transcript. To determine the structure of the GRP1 mRNA, PA^+ RNA was hybridized to an unlabeled GRP1 genomic subclone (labeled DNA-X) and then treated with S1. The denatured protected hybrids were electrophoresed, blotted to nitrocellulose, and hybridized to the ^{32}P-labeled insert of the same subclone used in the initial hybridization. If there is no intron in the region of the GRP1 DNA contained in the subclone, this procedure would result in a single band on the autoradiogram (A). This single band represents both the protected RNA and DNA hybrid. If, however, this region of DNA contains a single intron (B), three bands would be apparent on the autoradigoram. The top band represents the protected RNA fragment, while the lower two bands represent the protected DNA fragments. Analysis of the sizes of the protected RNA and DNA fragments from several experiments with various subclones of the GRP1 gene, in a manner analogous to restriction enzyme mapping, allows the determination of the ends of the GRP1 transcript and the location and size of any introns contained within the GRP1 genomic DNA.

scription of the GRP1 gene begins 46 bp upstream from the ATG initiation codon.

Detailed S1 analysis was also performed to confirm (Fig. 6) that the GRP1 transcript extended past the 3'-most *Hin*dIII site. This study was performed with RNA isolated from wounded stems and leaves hybridized to the 142-bp *Nde*I-*Hin*dIII fragment labeled at the 3' end of the *Nde*I site. This fragment was completely protected (Fig. 7), indicating that the GRP1 mRNA does extend through the *Hin*dIII site.

DISCUSSION

The data presented here show that there is only one transcriptional product for the GRP1 gene, a 1,600-b PA^+ mRNA, and that this gene is expressed in leaves, stems, and flowers but its expression is undetectable in roots. In addition, the steady-state level of the GRP1 mRNA was greater in young tissue than in old. The observed pattern of GRP1 expression is quite different from that of a tomato HRGP (extensin) gene, Tom 5 (22). This HRGP gene is expressed at much higher levels in roots and stems than in leaves and is higher in old tissue than in young tissue, especially in areas of lignification (A. Showalter, personal communication). These data lead us to predict that the petunia GRP1 gene product is more likely to be associated with the early development of the primary cell wall and probably does not function in a manner that imparts rigidity to the cell wall.

GRP1 and PAL mRNAs accumulate within 5 min after wounding, suggesting that the wound signal transduction system is in place prior to wounding and that there are few intervening steps between the original signal and the accumulation of the two mRNAs in petunia. It further indicates that the immediate accumulation of these RNAs is not dependent on transcriptional induction and subsequent expression of any other gene product. The possibility exists that wounding could induce the translational expression of an already transcribed but sequestered mRNA. The protein product of this mRNA could then induce the accumulation of

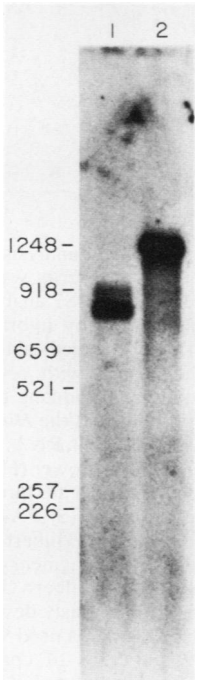

FIG. 6. S1 nuclease mapping of the GRP1 transcript PA^+ RNA (10 μg) was hybridized to *Sph*I- and *Pst*I-cleaved p7Sph-Pst (lane 1) or *Hin*dIII-cleaved p7D3-1200 (lane 2). Hybrids were treated with S1, and the resultant hybrids were subjected to RNA-DNA blot hybridization (see the legend to Fig. 5 and Materials and Methods). Blot hybridization was to ^{32}P-labeled p7D3-1200 and p7Sph-Pst. Numbers to the left indicate sizes of DNA standards run in parallel (in bases).

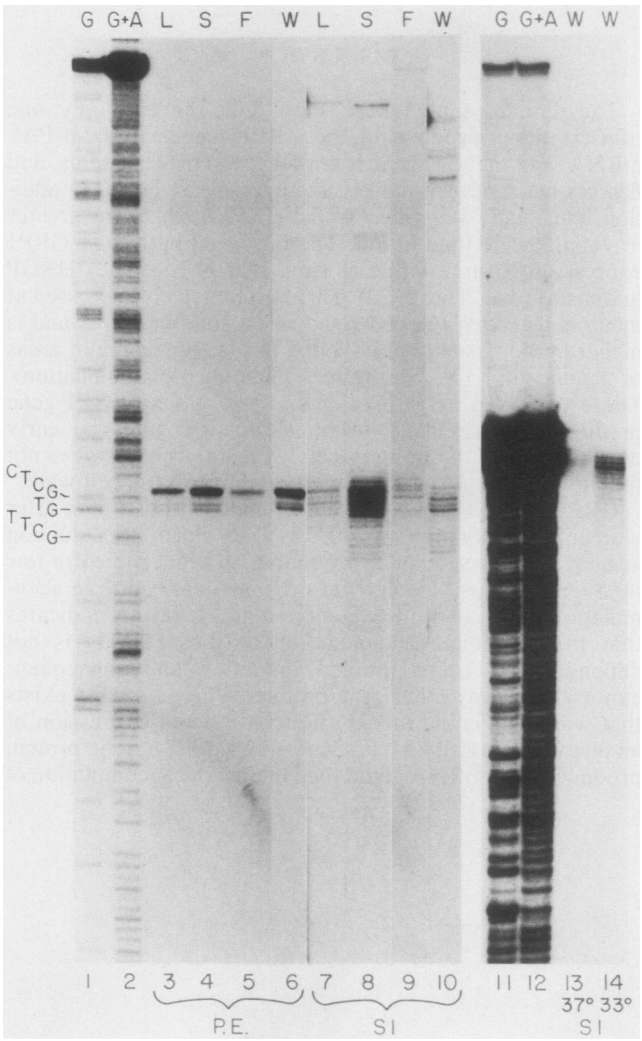

FIG. 7. Primer extension and S1 analysis of the GRP1 transcript. (Left) The 5′ end of the GRP1 transcript was determined by primer extension (P. E.; lanes 3 to 6) and S1 analysis (S1, lanes 7 to 10). Primer extension was performed by hybridizing the 46-bp *Xho*I-*Hin*dIII fragment of p7D3-500 labeled at the *Hin*dIII site to PA^+ RNA and extending the hybrid with reverse transcriptase. S1 digestion was performed after hybridizing the 246-bp *Sph*I-*Hin*dIII fragment of p7D3-500, ^{32}P-labeled at the *Hin*dIII site, to PA^+ RNA. Lanes 3 and 7, 10 μg of leaf (L) PA^+ RNA; lane 4, 5 μg of stem (S) PA^+ RNA; lanes 5 and 9, 20 μg of flower (F) PA^+ RNA; lanes 6 and 10, 5 μg of PA^+ RNA isolated from a mixture of wounded leaves and stems (W); lane 8, 10 μg of stem (S) PA^+ RNA. Lanes 1 and 2 represent, respectively, Maxam and Gilbert G and G+A sequencing tracks of the 246-bp *Sph*I-*Hin*dIII fragment of p7D3-500, ^{32}P-labeled at the *Hin*dIII site. Maxam and Gilbert G and G+A sequencing reactions were performed as previously described (21). The specific activity of the end-labeled fragments used was 1.3×10^6 cpm/pmol (lanes 3, 4, 5, 7, 8, and 9) and 0.5×10^6 cpm/pmol (lanes 6 and 10). Autoradiography was for 2 days (lanes 3, 4, 5, 7, 8, and 9) and 12 h (lanes 6 and 10). (Right) S1 analysis of the 3′ end of the GRP1 transcript. S1 digestion was performed after hybridizing the 142-bp *Nde*I-*Hin*dIII fragment of p7D3-1200, ^{32}P-labeled at the *Nde*I site to 5 μg of PA^+ RNA isolated from wounded leaves and stems. Lane 13, Hybridization performed at 37°C; lane 14, hybridization performed at 33°C. Lanes 11 and 12 represent, respectively, Maxam and Gilbert (21) G and G+A sequencing tracks of the 142-bp *Nde*I-*Hin*dIII fragment, ^{32}P-labeled at the *Nde*I-site.

GRP1 and PAL mRNAs. Alternatively, it is possible that a change in intracellular ion concentration could create the rapid signal necessary for accumulation of the two mRNAs. It is likely that plants contain signal transduction systems similiar to those described for animal cells, in which changes in intracellular ion concentrations appear to affect the expression of genes (3, 12, 16). Intracellular ion concentrations do change in plants in response to stimuli. It has been known for some time that auxin, a plant hormone which promotes growth and elongation of cells, causes an H^+ efflux from the cell (27). It has also been shown that light acts as a primary signal in plants, causing a cytosolic decrease in Ca^{2+} concentration and an increase in chloroplastic Ca^{2+} concentration (12, 13, 17). Additionally, calcium antagonists and calmodulin inhibitors have been shown to block cytokinin-induced bud formation in moss (20). Thus, although primary signals, receptors, and secondary mRNAs may differ between animal and plant cells, intracellular ion concentrations of plant cells may also affect plant gene expression.

In contrast to the rapid accumulation of GRP1 and PAL transcripts we observed in petunia, accumulation of PAL mRNA in French bean is not discernable until 2 h after wounding (2). This difference may indicate a different temporal arrangement in the events of the wound response in the two species. It may also indicate that certain events necessary for the wound activation of the PAL gene in bean are not necessary or are already in place in petunia prior to wounding. Wounding is one of the mechanisms by which pathogens gain entry to the plant and establish infection. Thus, in nature, the wound and defense responses are intimately connected. It has been shown that some genes which are activated by elicitor (PAL, CHS [chalcone synthase], and HRGP) as part of the plant defense mechanism are also at least partially activated by wounding (2, 9, 15, 22). Furthermore, it has been shown that one difference between an incompatible (host-resistant) plant pathogen interaction and a compatible (host-susceptible) interaction is that plant defense gene transcripts accumulate much earlier in the incompatible reaction (2). It would be extremely useful to determine whether the entire process of the wound response actually occurred in an earlier time frame in petunia than in bean or whether the earlier accumulation of mRNA we observed was due to technological differences in our experiments. If there is a real difference, comparative studies of the wound response in bean and petunia could provide information about the mechanisms that allow earlier activation of defense genes. Application of these mechanisms to other plant species might provide a basis for the enhancement of plant disease resistance.

We have shown in this paper that the pattern of GRP1 RNA expression is highly variable. Such diversity of expression argues that regulation of this gene is highly complex, especially since the same transcriptional start site was used in all organs and under all conditions of induction. The rapid and extensive enhancement in the levels of GRP1 mRNA after wounding, especially in old tissue, suggests that the expression of this gene is at least partially controlled at the level of transcription. Other workers have recently shown that PAL, CHS, and HRGP genes in bean are all transcriptionally activated by wounding (15) and have proposed that all plant defense mechanisms involve the transcriptional activation of these genes. If the control of expression of the GRP1 gene follows the pattern of other plant defense genes, an analysis of sequences flanking the GRP1 gene should provide information of organ-specific, development-specific, and defense-specific expression of genes in plants.

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