Differential Expression of Five Arabidopsis Genes Encoding Glycine-Rich Proteins

Dulce E. de Oliveira, Jef Seurinck, Dirk Inzé, Marc Van Montagu and Johan Botterman

Five cDNA clones coding for glycine-rich proteins in Arabidopsis thaliana were isolated. The corresponding genes are present in the genome as single copies. The derived protein sequences contain highly repetitive glycine-rich motifs. There is, however, little homology among them, nor with previously described glycine-rich proteins from other species. All five genes are expressed in leaves and stems of 6-week-old plants but show different patterns of expression in other organ systems. Analysis of the effect of different external stimuli on the expression pattern showed that, in most cases, the transcript levels were moderately but selectively affected. With flooding stress, the accumulated level of the transcript from one of the genes was remarkably increased.

INTRODUCTION

Glycine is a major fraction of the total protein nitrogen in certain tissues. Examples are the soybean and gourd seed coat with 21% of glycine and the cell wall of milkweed stem and of coat coleoptile cells with 31% and 27% of glycine, respectively (Varner and Cassab, 1986). The fact that the cell wall contains several proteins with a normal content of glycine (8% to 12%) and structural proteins with no or very few glycine residues such as extensin (Chen and Varner, 1985) or other (hydroxy)proline-rich proteins (Cooper et al., 1987; Hong et al., 1987; and Datta et al., 1989) implies that glycine-rich proteins should also be present. For a review of cell wall proteins, see Cassab and Varner (1988). Such glycine-rich proteins (GRP) have indeed been isolated from the pumpkin seed coat (Varner and Cassab, 1986) and strawberry fruit (Reddy and Poovaiah, 1987) with 47% and 49% glycine residues, respectively.

Genes encoding GRPs have been isolated from petunia (Condit and Meagher, 1986), bean (Keller et al., 1988), maize (Gómez et al., 1988), and tobacco (van Kan et al., 1988). Transgenic tobacco plants expressing β-glucuronidase under control of the promoter of the bean grp1.8 gene showed induction in a specific set of cells close to the site of wounding (Keller et al., 1989a). Abscisic acid and water stress induce grp expression in maize. The tobacco gene is induced by salicylic acid, light, and virus infection (van Kan et al., 1988). Light also induces a grp gene in pigweed (Kaldenhoff and Richter, 1989).

We describe the isolation and characterization of different cDNA clones representing a set of grp-like genes in Arabidopsis thaliana. They encode proteins with unusual patterns of glycine-rich repeats, which differ from previously described grp genes. They are present as single copy genes in the genome of Arabidopsis, show different patterns of organ-specific expression, and display different responses to external stimuli.

RESULTS

Isolation of grp cDNA Clones and Gene Expression Pattern under Standard Growth Conditions

A genomic clone from A. thaliana, which shows inflorescence-specific expression, was isolated by differential screening (Simoens et al., 1988). Characterization of this...
clone revealed sequences coding for glycine-rich polypeptides (D.E. de Oliveira, unpublished results). Riboprobes made from these glycine-rich coding sequences were used to screen a plasmid-based cDNA library of Arabidopsis. Approximately 50 positive cDNA clones were identified by colony hybridization. They were grouped in five classes based upon restriction patterns and DNA gel blot analysis (data not shown). The largest clone found in each of the five classes was designated as atGRP-1, -2, -3, -4, and -5 and was chosen for further characterization.

As shown in Figure 1, antisense RNA probes from the respective clones hybridized with transcripts of 1.1 kb, 1.2 kb, 0.7 kb, 0.65 kb, and 0.8 kb, respectively. At high stringency the probes were transcript specific and no cross-hybridization was observed. Because the complementary strand of repetitive glycine (GGN) codons consists of proline (CCN) codons, hybridizations were also performed with riboprobes prepared from the alternative strand of the cDNA. In this case, no signals were detected in RNA gel blot analysis using the same hybridization conditions (data not shown).

The five clones showed different patterns of expression in individual organ systems from 6-week-old Arabidopsis plants (Figure 1). atGRP-1 and -2 transcripts were found in roots, stems, leaves, and seed pods, and in the inflorescence, although at a different level. The accumulation of atGRP-1 mRNA was clearly greater in stems than in the other tissues analyzed, whereas the atGRP-2 transcript was about equally abundant in all organs. atGRP-3 was mainly expressed in stems and leaves. atGRP-4 mRNA was present at low levels in stems and leaves, and was detectable in flowers only after prolonged exposure. In such exposures, hybridization with RNA of less than 0.25 kb was also observed in flowers, which could be due to the presence of transcripts with homologous sequences or to degradation of the atGRP-4 mRNA. atGRP-5 was more abundantly expressed in immature seed pods than in stems and leaves, and its expression was barely detectable in flowers and roots.

**grp Sequences in the Arabidopsis Genome**

The patterns of hybridization of the five atGRP cDNAs to a DNA gel blot of total Arabidopsis DNA are shown in Figure 2. Hybridization at moderate stringency with atGRP-1 as probe revealed several fragments (Figure 2A) suggesting the existence of other related sequences in the genome of Arabidopsis. Such sequences could be related to GRPs, hydroxyproline-rich glycoproteins, or proline-rich proteins, similar to what has been observed in bean (Keller et al., 1988). At high stringency, different patterns of hybridization were observed for the five different cDNA clones (Figure 2B). In all cases, only one or two bands were detected. These results, together with the genomic reconstruction (Figure 2B) and the sequencing data shown below, lead to the conclusion that each cDNA clone represents a different gene, which is present as a single copy per genome equivalent, or as a few copies in the case of atGRP-2.

**Nucleotide Sequence of the grp cDNA Clones**

The nucleotide sequence of the cDNAs is given in Figures 3 to 7. Only one plausible open reading frame (ORF) is identified in the coding strand of the cDNAs atGRP-2 to -5. For atGRP-1, one additional ORF basically consisting of hydrophilic amino acids is found, which would yield a highly improbable protein. On the other hand, the glycine-rich protein sequence derived from this nucleotide sequence fits perfectly with the structure models for GRPs. Therefore, we concluded that these genes code for proteins with a significant number of glycine residues. In all cases, the 3' portion of the genes is complete because putative polyadenylation signals followed by a polyA tail are found. However, 5' sequences may be absent because the length of the insert does not always match the size of the transcript estimated from RNA gel blot analysis (Figure 1).

**Figure 1. Expression of the atGRP Transcripts in Roots (R), Stems (S), Leaves (L), Immature Seed Pods (SP), and Flowers (F) of *A. thaliana*.**

The RNA gel blot (5 μg of total RNA per lane) was hybridized to 32P-labeled antisense RNA of atGRP-1, -2, -3, -4, and -5. Transcript sizes (kb) estimated by comparison with RNA size standards (Bethesda Research Laboratories) are indicated.
Glycine-Rich Proteins from Arabidopsis 429

H B H B bp -11495 -<507 I -2836 -1700 [atGRP-1 | atGRP-t atGRP-2 atGRP-3 atGRP-4 atGRP-5 genomic reconstr.

Figure 2. DNA Gel Blot Analysis of atGRP Sequences in the Arabidopsis Genome.

Total DNA from leaves of Arabidopsis was digested with HindIII (H) and BgIII (B). The fragments were separated by electrophoresis in 0.75% agarose gels and blotted on nylon membrane.

(A) Hybridization with a 32P-labeled atGRP-1 riboprobe under conditions of moderate stringency.

(B) Hybridization with 32P-labeled riboprobes of the different atGRPs (as indicated at bottom) under high-stringency conditions. Genomic reconstruction lanes contain EcoRI-digested atGRP-1 cloned in pGEM1 as one (1) and five (5) copies per genomic equivalent, which is based on a size of 7.0 x 10^4 kb per haploid genomic equivalent (Pruitt and Meyerowitz, 1986). The length of the λ DNA size markers resulting from a PstI digest are indicated on the right.

atGRP-2, -3, and -5 apparently contain the entire open reading frame.

The atGRP-1 gene is represented in Figure 3A. The G content of the ORF is high (54%) compared with the 3' untranslated region (21%). The translated amino acid sequence, of which the 5' end is missing, contains 210 amino acids with 75% glycine residues. It largely consists of (Glyn-Ala-Gly)n-Phe/His stretches. The hydropathic index, according to Kyte and Doolittle (1982), revealed that atGRP-1 is highly hydrophobic (Figure 3B), and the secondary structure prediction, according to Garnier et al. (1978), gives long stretches of extended conformation.

Such a structural pattern fits well with the model of antiparallel strands forming a β-pleated sheet (Condit and Meagher, 1986; Keller et al. 1988).

In the atGRP-2 cDNA, a 5'-untranslated region of 55 nucleotides; a complete ORF with 40% G content, coding for a protein consisting of 40% glycine residues; and a 3' nontranslated region can be identified, as shown in Figure 4A. The putative initiation codon is in frame with an upstream stop codon. The predicted protein sequence has two separate glycine-rich segments, and the hydropathic plot shows both hydrophilic and hydrophobic regions (Figure 4B). The N-terminal region is hydrophilic, and no trans-

Figure 3. Primary Structure and Hydropathic Index of the atGRP-1 cDNA Clone.

(A) The nucleotide sequence is represented and aligned with the amino acid sequence of the putative encoded protein. The stop codon is represented by a dot. The putative polyadenylation signal is underlined. Amino acid residues different from Gly or Ala are underlined.

(B) Hydropathic index of the predicted amino acid sequence, computed using an interval of 9 amino acids. The hydropathicity is represented in function of the amino acid number. The dotted line at the -5 value represents the midpoint line, with hydrophobic domains above and hydrophilic domains below.
Figure 4. Primary Structure and Hydrophobic Index of the atGRP-2 cDNA Clone.

(A) The nucleotide sequence and amino acid sequence of the putative encoded protein are represented as described in Figure 3A. The arrows mark the boundaries between glycine-rich and glycine-poor domains. The nonglycine residues in the glycine-rich domain are underlined.

(B) Hydrophobic index of the predicted amino acid sequence is represented as described in Figure 3B. The two intervals indicated by the bars are glycine-rich segments.

Figure 5A represents the 3' portion of the atGRP-4 gene. The ORF is composed of 35% G residues, whereas the 3'-nontranslated region only contains 15%. The predicted polypeptide shows a repeated sequence composed of a hydrophobic glycine-rich domain and a nonglycine-rich domain (Figure 5B).

The nucleotide sequence of atGRP-5 contains an ORF with 45% G residues, as shown in Figure 7A. The nontranslated 3' end has 20% G residues. As with atGRP-3, the N-terminal portion of the predicted protein resembles the signal peptide consensus sequence. Glycine represents 58% of the total amino acid composition. The glycine-rich segment is highly hydrophobic (Figure 7B) and can be summarized as (Gly$_n$,-X)$_m$ where $n$ is an odd number and $X$ = Leu, Ile, Ser, Phe, Ala, or His. Secondary structure prediction indicates an initial transmembrane helix, followed by long hydrophobic segments of extended structure, alternating with a few coils or turns. As for atGRP-1 and -3, it can be proposed that the glycine-rich domain of the atGRP-5 protein consists of antiparallel strands arranged in a $\beta$-pleated sheet configuration.

Comparison of the individual protein-encoding sequences revealed weak homology among them and with other data base sequences. However, the alignment of glycine-rich segments of atGRP-1 and -5 indicated an overall homology of 70%, similar to the glycine-rich domain of the bean GRP1.8 and petunia GRP. This apparent homology is expected because these four genes have stretches of glycine composed of Gly$_n$-X, with $n$ being an odd number.

Differential Accumulation of grp Transcripts in Response to External Stimuli

The atGRP cDNAs were used as hybridization probes to monitor changes in the levels of the corresponding tran-
scripts in 4-week-old Arabidopsis plantlets exposed to ethylene, salicylic acid, abscisic acid, water, and drying. The results are shown in Figure 8. The RNA levels were measured at different timepoints after applying the stimulus.

Under the experimental conditions used, only moderate or no effect on the accumulation of the transcript levels was observed. atGRP-4 transcripts were not detectable in unstressed 4-week-old plantlets, and no induction was

---

**Figure 5. Primary Structure and Hydropathic Index of the atGRP-3 cDNA Clone.**

(A) The nucleotide sequence and amino acid sequence of the putative encoded protein are represented as described in Figure 3A. Arrowheads mark hydrophobic and nonpolar amino acids that lie within a putative signal peptide sequence. The arrows mark the boundaries between glycine-rich and glycine-poor domains. The nonglycine residues in the glycine-rich domain are underlined. (B) Hydrophobic index of the predicted amino acid sequence is represented as described in Figure 3B. The interval indicated by the bar is the glycine-rich segment.
found upon stress (data not shown). With each external stimulus, the patterns of accumulation for the individual GRP transcripts were different. For example, salicylic acid had a different impact on the levels of all atGRP mRNAs. The atGRP-1 transcripts were increased threefold after 12 hr but were again reduced after 48 hr. The accumulation of atGRP-2 and -5 mRNA was higher after 24 hr and 48 hr, respectively. In contrast, the levels of atGRP-3 mRNA were increased continuously with time after application of salicylic acid. Upon drying, the levels of atGRP-5 were stable, whereas for atGRP-3, a moderate increase was observed after 6 hr, followed by a reduction after 12 hr. The levels of atGRP-1 and -2 transcripts were dramatically reduced under these conditions. Ethylene and abscisic acid (ABA) stimulated moderately the accumulation of the atGRP-3 mRNA.

More striking responses of atGRP-5 were observed when plantlets were submitted to ABA or water. During the ABA treatment, the level of the atGRP-5 transcript increased threefold, but a more remarkable increase was observed when the plantlets were transferred to a filter paper saturated with water. After 24 hr of incubation in water, the atGRP-5 mRNA level was increased sevenfold relative to unstressed plantlets. Because the ABA treatment was performed by transfer of the plantlets to a filter paper saturated with water (lanes W).

Six micrograms of total RNA isolated at the given timepoints (hours) after the application of the stress were applied in each lane. Lane 0, control (before treatment); lanes E, ethylene; lanes S, salicylic acid; lanes A, abscisic acid; lanes D, drying; lanes W, water. The bar graph represents the relative quantification of the mRNA levels of atGRP-5. The data were normalized relative to the mean value from six different control samples. These samples were obtained from nontreated plantlets randomly applied on the gel. The first lane corresponds to one of the controls. In the case of ABA treatment, which was performed by transferring the plantlets to filter paper saturated with a solution of ABA (see Methods), the controls were the plantlets transferred to filter paper saturated with water (lanes W).
paper saturated with a solution of ABA, the difference in the accumulated levels of atGRP-5 mRNA relative to control plants should be attributed to the flooding stress. The presence of ABA appears to partially abolish this effect.

**DISCUSSION**

We have characterized five different cDNAs from *Arabidopsis thaliana* encoding glycine-rich proteins (atGRP). These clones were isolated using a genomic fragment containing genes that are preferentially expressed in the inflorescence (Simoens et al., 1988; D.E. de Oliveira, unpublished results). None of the atGRP cDNAs corresponds to the inflorescence-specific genes nor to grp genes previously described in other species. The cDNA library screened was derived from total plant mRNA (Alliotte et al., 1989), which, therefore, is abundant in cDNAs representing stem and leaf transcripts. Indeed, RNA gel blot analysis showed that the isolated cDNAs were all expressed in stems and leaves, although they showed different patterns of expression in other organs (Figure 1).

The DNA gel blot hybridization at less stringent conditions (Figure 2A), the identification of five different grp genes described here, and the three grp genes with preferential expression in flowers (data not shown) indicate the existence of multiple glycine-rich encoding genes in *A. thaliana*. Some of their derived cDNAs were not present in the library analyzed. This is not surprising because glycine-rich genes may be highly regulated, both in terms of organ specificity and developmental profile. The latter case has been observed with grp genes from other plant species that are mostly expressed in young tissues (Condit and Meagher, 1986; Gómez et al., 1988; Keller et al., 1988).

The isolated cDNAs all contain an ORF with a high G content (34% to 54%), and glycine is the most abundant amino acid of the encoded proteins (31% to 75%). In two clones, atGRP-3 and -5, N-terminal sequences resembling signal sequences for protein transport were observed. In two other cases, atGRP-1 and -4, the ORF was incomplete, and, in one example (atGRP-2), the primary structure did not reveal any transmembrane peptide. This suggests that these glycine-rich proteins may have different cellular locations. Although most of the GRPs previously described are thought to be components of cell walls, a protein with high content of glycine residues has also been found in the soluble fraction of rice leaf extracts (Mundy and Chua, 1988).

The *Arabidopsis* GRPs described here show very distinct primary structures, although all contain characteristic repetitive structural motifs. The nonglycine residues present in the repeats vary greatly, yielding a more hydrophobic or hydrophilic polypeptide chain (Figures 3B, 4B, 5B, 6B, and 7B). The atGRP-1 polypeptide contains, besides Gly and Ala, predominantly Phe and His (9.8%), which are also present in significant amounts in other GRPs (Condit and Meagher, 1986; Keller et al., 1988). Histidine is also abundant in hydroxyproline-rich glycoproteins (HRGP) and may be involved in interactions with other cell wall components (Fry, 1986; Cooper et al., 1987).

The glycine-rich domain of all five *Arabidopsis* GRPs is predicted to assume an extended conformation. The cloned fragment of atGRP-1 fits well with the model of antiparallel strands forming a β-pleated sheet. Assuming this configuration, the histidine residues would be positioned on the outer edge of the structure and the β-pleated sheet would be formed by odd numbers of Gly intercalating with Ala, Phe, Leu, or Gin. This distribution allows the exposition of the bulky side chains to one side of the sheet, whereas on the other side stay only hydrogen atoms of glycine residues. The glycine-rich sequence of atGRP-5 reveals similar configuration, with the bulky side chains projected to one side of the β-pleated sheet. This model is like the proposed structure of GRPs from petunia and bean (Condit and Meagher, 1986; Keller et al., 1988). This arrangement of glycine residues might, thus, define a precise structure in these molecules.

The atGRP-3 protein is hydrophilic and has high levels of tyrosine (8.7%) regularly arranged in the glycine-rich domain. A β-pleated sheet with seven antiparallel strands can be formed from this glycine-rich amino acid sequence. Assuming this configuration, the amino acid residues would be arranged in the following way:

\[
\begin{align*}
G_6 & G_2 Y \quad G_6 G_2 Y \quad G_6 \\
G_4 & G_2 Y \quad G_6 G_2 Y \quad G_6 \\
G_6 & G_2 Y \quad G_6 G_2 Y \quad G_6 \\
G_8 & G_2 Y \quad G_6 G_2 Y \quad G_6 \\
G_{10} & G_2 Y \quad G_6 G_2 Y \quad G_6 \\
G_{12} & G_2 Y \quad G_6 G_2 Y \quad G_6 \\
G_{14} & G_2 Y \quad G_6 G_2 Y 
\end{align*}
\]

Two glycine residues of the Gly, stretch would lie on the outside of the sheet, with Tyr on one side and Gin, Asp, and Asn on the other side of the plane made by the sheet. The proposed structure results in a perfectly symmetrical arrangement of identical side chains.

Tyrosine is also typical in extensin and other HRGPs (Corbin et al., 1987). Peroxidases can form intra- and intermolecular cross-links between tyrosine residues, resulting in an HRGP network that serves as matrix for the linkage of polysaccharides or polyphenols (Epstein and Lamport, 1984; Fry, 1986; Bol and van Kan, 1988). A high content of regularly arranged tyrosines is also present in GRPs from maize and bean (Gómez et al., 1988; Keller et al., 1988). Because of the close correlation between the accumulation of the bean protein with the pattern of lignification, tyrosine residues were suggested to be substrates for initiation of the polymerization chain reaction for lignin synthesis. Different GRPs with specific tyrosine arrays may lead to different lignin structures in terms of density and three-dimensional pattern.
Arabidopsis thaliana (C24) was grown in the greenhouse or in the culture room (22°C to 24°C). (5) water: plantlets were transferred to 3MM Whatman paper saturated with sterile distilled water. Upon harvesting, the samples were frozen in liquid nitrogen and stored at −70°C.

A. thaliana cDNA Library

The construction of the cDNA library from polyA+ RNA isolated from total plants has been described previously (Alliotte et al., 1989). The screening was performed with riboprobes according to standard techniques (Maniatis et al., 1982).

DNA and RNA Preparation

Total Arabidopsis DNA was prepared according to Pruitt and Meyerowitz (1986). Total RNA from different organs of Arabidopsis was prepared according to Jones et al. (1985). For detection of grp transcripts under stress conditions in different organ systems. RNA was isolated from Arabidopsis plantlets in small quantities, according to Verwoerd et al. (1989).

Nucleic Acid Hybridizations

DNA gel blot analysis was performed according to Maniatis et al. (1982), using Hybond N (Amersham) membranes and following the recommendations of the manufacturer. Total RNA was de-

tured with glyoxal and dimethyl sulfoxide according to Mc-

主 and Carmichael (1977), fractionated by agarose gel elec-

trophoresis, and transferred to Hybond N membranes. 32P-labeled RNA was synthesized using the in vitro transcription Riboprobe Gemini System (pGEM1, Promega Biotec). Hybridizations of probes to DNA and RNA blots were performed in 50% formamide, 0.5% SDS, 0.1 mg/mL denatured salmon sperm DNA, 0.25% lyophilized low-fat milk, and 5 x SSC for DNA blots or SSPE for RNA blots. The filters were prehybridized for 4 h and hybridized overnight at 42°C or 65°C for DNA and RNA gel blots, respectively. The DNA blots were washed twice for 30 min with each of the following solutions: 3 x SSC, 1% SDS; 1 x SSC, 1% SDS; 0.1 x SSC, 0.1% SDS at 42°C; and 0.1 x SSC, 0.1% SDS at 65°C for stringent conditions. At less stringent conditions, the minimum salt concentration was 0.5 × SSC, 0.1% SDS at 42°C and 60°C. The RNA blots were washed at 68°C using the solutions described for stringent DNA gel blot hybridizations. Subsequently, the filters were briefly air dried and autoradiographed with XAR-films (Kodak) using intensifying screens.

Quantification of mRNA

After RNA gel blot hybridization, the membranes were exposed to pre-flashed XAR films (Kodak). The relative amounts of mRNA were determined by densitometric scanning of the autoradiographs with an Ultroscan Laser Densitometer (LKB model 2202).
Nucleic Acid Sequencing and Analysis

DNA sequences were determined according to Maxam and Gilbert (1980). The derived amino acid sequences were aligned with GENALIGNS using the region program (copyright of Intellegenetics, Inc.). The percentage of homology was calculated as the ratio of the aligned amino acids to the length of the region showing significant alignment. Hydropathic plots were made using PC/GENE (copyright Dominique Garin). DNA and protein sequences were used to search for homology with sequences present in the National Institutes of Health Nucleic Acid Sequence Databank (GENBANK), The University of Geneva Protein Data Bank (SWISS-PROT), and The Protein Identification Resource of the National Biomedical Research Foundation (PIR).

ACKNOWLEDGMENTS

We thank Drs. Enno Krebbers and Nadir Mrabet for scientific discussion and critical reading of the manuscript, and Karel Spruyt and Vera Vermaercke for preparing the illustrations. Dulce E. de Oliveira was supported by the Brazilian Research Council (CNPq) and European Economic Community Fellowships.

Received December 21, 1989; revised March 13, 1990.

REFERENCES


Differential expression of five Arabidopsis genes encoding glycine-rich proteins.
D E de Oliveira, J Seurinck, D Inzé, M Van Montagu and J Botterman
*Plant Cell* 1990;2:427-436
DOI 10.1105/tpc.2.5.427

This information is current as of October 20, 2017