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A Novel Extensin Gene Encoding a Hydroxyproline-Rich Glycoprotein Requires Sucrose for Its Wound-Inducible Expression in Transgenic Plants

Ji Hoon Ahn,[a,b] Yeonhee Choi,[a,b] Young Myung Kwon,[b,c] Sang-Gu Kim,[b,c] Yang Do Choi,[d] and Jong Seob Lee[a,b,1]

[a] Department of Molecular Biology, Seoul National University, Seoul 151-742, Korea
[b] Research Center for Cell Differentiation, Seoul National University, Seoul 151-742, Korea
[c] Department of Biology, Seoul National University, Seoul 151-742, Korea
[d] Department of Agricultural Chemistry, Seoul National University, Suwon 441-744, Korea

A novel hydroxyproline-rich glycoprotein (SbHRGP3) that consists of two different domains is encoded by an extensin gene from soybean. The first domain (domain 1) located at the N terminus is composed of 11 repeats of Ser-Pro_4-Lys-His-Ser-Pro_4-Tyr_3-His, whereas the second domain (domain 2) at the C terminus contains five repeats of Ser-Pro_4-Val-Tyr-Lys-Tyr-Lys-Ser-Pro_4-Tyr-Lys-Tyr-Pro-Ser-Pro_5-Tyr-Lys-Tyr-Pro-Ser-Pro_4-Val-Tyr-Lys-Tyr-Lys. These two repeat motifs are organized in an extremely well-ordered pattern in each domain, which suggests that SbHRGP3 belongs to a new group of proteins having the repeat motifs of two distinct groups of dicot extensins. The expression of the *SbHRGP3* gene increased with seedling maturation, and its expression was relatively high in the mature regions of the hypocotyl and in the root of soybean seedlings. An *SbHRGP3*-β-glucuronidase (*SbHRGP3-GUS*) chimeric gene was constructed and expressed in transgenic tobacco plants. The expression of the *SbHRGP3-GUS* gene was not induced by wounding alone in transgenic tobacco plants; sucrose was also required. Expression was specific to phloem tissues and cambium cells of leaves and stems. In transgenic tobacco seedlings, *SbHRGP3-GUS* gene expression was activated by the maturation of the primary root and then inactivated; however, reactivation was specifically at the epidermis of the zone from which the lateral root was to be initiated. Its reactivation occurred just before the lateral root initiation. These results indicate that the *SbHRGP3* gene in different tissues responds to different signals.

INTRODUCTION

The plant cell has a rigid extracellular framework called the cell wall. Plant cell walls have not only structural roles, such as that of mechanical support, but also important physiological functions, such as transport, absorption, and secretion of substances (Lamport, 1965). The precise molecular composition and structure of cell walls depend on the cell, tissue, and plant species (Bacic et al., 1988; Carpita and Gibeaut, 1993).

To date, five classes of proteins that are abundant in cell walls have been studied in various plants (reviewed by Showalter, 1993). These are hydroxyproline-rich glycoproteins (HRGPs), glycine-rich proteins, proline-rich proteins (PRPs), solanaceous lectins, and arabinogalactan proteins. All of these classes of proteins may be evolutionarily and functionally related to each other because they are enriched in hydroxyproline residues or share nucleotide sequence similarity.

Extensins are a family of HRGPs and constitute the major protein components in cell walls of dicot plants (Showalter, 1993). The distinctive characteristic of dicot extensins is their repetitive Ser-Pro_4 pentapeptide blocks. These pentapeptide blocks are conserved in almost all dicot extensins (Chen and Varner, 1985; Smith et al., 1986; Showalter and Varner, 1989) and have been reported in a gymnosperm extensin (Fong et al., 1992). Extensins are very rich in Pro and Ser and in combinations of Val, Tyr, Lys, and/or His. Extensins are basic proteins with high isoelectric points probably due to a high content of Lys and/or His, depending on the molecule. It has been proposed that extensins have two functions in plants (Wilson and Fry, 1986). First, they may contribute to the structural support of the cell wall by forming glycoprotein networks, even though direct functional evidence is absent. However, Qi et al. (1995) recently showed some evidence that extensins cross-link pectins in cell walls. Second, they contribute to plant defense, helping to protect against pathogen attack or mechanical wounding (Showalter, 1993). There is some evidence that extensins may act as impenetrable physical barriers or may immobilize the pathogens by binding to their surfaces (Mazau et al., 1987). The latter probably results from positively charged extensin molecules interacting ionically with negatively charged

[1] To whom correspondence should be addressed at the Department of Molecular Biology, College of Natural Sciences, Seoul National University, Seoul 151-742, Korea.

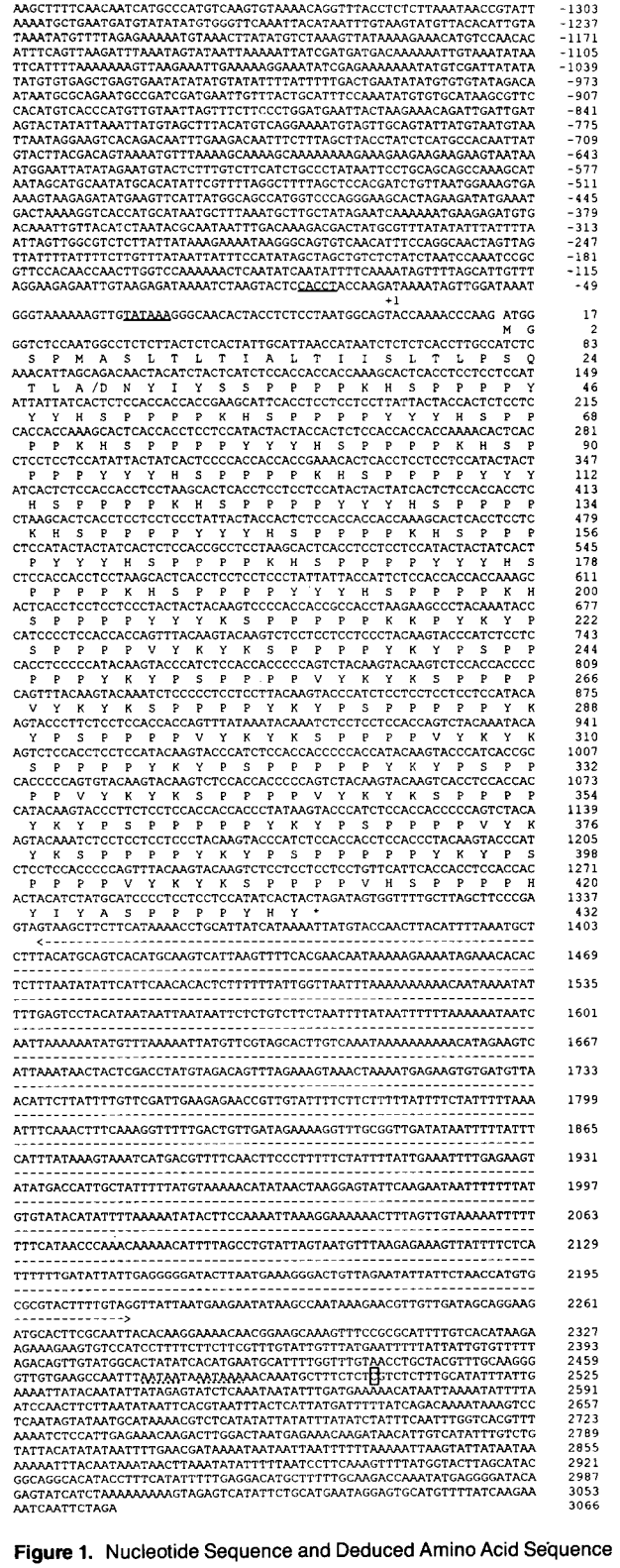

Figure 1. Nucleotide Sequence and Deduced Amino Acid Sequence of the *SbHRGP3* Gene.

surfaces of plant pathogens (Leach et al., 1982; Mellon and Helgeson, 1982).

Although extensins are synthesized as soluble precursors, the majority of Pro residues are hydroxylated, and most of both hydroxyproline and Ser residues are glycosylated by post-translational modifications (Kieliszewski and Lamport, 1994). Short arabinoside chains of one to four units in length are glycosidically attached to the hydroxyproline residues, and single galactosyl residues are attached to some Ser residues. When secreted to the cell wall, extensins become insoluble, presumably due to the formation of covalent cross-linkages, such as isodityrosine (IDT) bridges (Iiyama et al., 1994). The functional sites involved in various aspects of cell wall assembly have been suggested to include binding sites, signal peptides, helix domains, β strands and sheets, and coil segments (see Kieliszewski and Lamport, 1994).

Various extensin genes have been isolated from many plant species, including carrot (Chen and Varner, 1985), bean (Corbin et al., 1987), tobacco (Keller and Lamb, 1989), tomato (Showalter et al., 1991), potato (Bown et al., 1993), monocots (Li et al., 1990; Kieliszewski et al., 1992), and a gymnosperm (Fong et al., 1992). Expression of the extensin genes is not constitutive but rather is induced in response to mechanical wounding, pathogen attack, or ethylene treatment (Corbin et al., 1987; Memelink et al., 1993; Parmentier et al., 1995; Wycoff et al., 1995) and is developmentally regulated (Keller and Lamb, 1989; Ye and Varner, 1991). Their expression is specific to tissues, such as phloem and cambium tissues in healthy plants (Showalter, 1993).

In soybean, the expression of *HRGP* genes has also been identified and determined to be developmentally regulated in a tissue-specific manner (Ye and Varner, 1991). *HRGP* genes are most highly expressed in cambium cells of stems, and their expression has also been observed in petioles, seed coats, and young hypocotyls. Furthermore, it has been reported that PRPs are localized in a limited number of cell types of a particular organ in soybean (Wyatt et al., 1992). These data indicate that the cell wall proteins are different in each cell type.

In this study, we report the isolation of an extensin gene encoding a novel HRGP that has two different domains with a highly ordered array of repeats. We show that the expression of this extensin gene increases as seedlings mature. Interestingly, wound induction of the extensin gene in phloem tissues

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The amino acids of the encoded polypeptide are designated in the single-letter code. The asterisk indicates the translation stop codon. The putative CAAT and TATA boxes in the 5′ flanking region are underlined, and the putative poly(A) addition signals are underscored with dots. The poly(A) tail is added after the boxed C residue (+2505). The +1 denotes the transcription start site of the *SbHRGP3* mRNA. The slash between A and D indicates the putative cleavage site of the signal peptide. The sequence above the dashed line with arrowheads indicates an intron in the 3′ untranslated region. The GenBank, EMBL, and DDBJ accession number for the *SbHRGP3* nucleotide and predicted amino acid sequences is U44838.

and cambium cells of leaves and stems in transgenic tobacco plants also requires sucrose.

RESULTS

Structure of the *SbHRGP3* Gene

We have previously isolated a cDNA clone, SE126, that encodes an extensin in soybean (Choi, 1995). Using the SE126 cDNA as a probe, we isolated a genomic clone from a soybean genomic library. The nucleotide sequence of 4.4 kb around the extensin gene was determined (Figure 1). It shows a striking identity to that of a partial extensin cDNA clone of soybean, *SbHRGP3*, except for one nucleotide (T to C at position +1076) in the coding region (Hong et al., 1994).

The transcription initiation site of the extensin gene (*SbHRGP3*) was determined by primer extension analysis. As shown in Figure 2, the transcription of the *SbHRGP3* gene starts at an A residue, 13 nucleotides upstream of the translation start codon. Therefore, the *SbHRGP3* mRNA is considered to be 1636 bases long, excluding the poly(A) tail. The TATA box (positions −34 to −29) and CAAT box (positions −78 to −74) are located in the 5′ flanking region, and two putative poly(A) addition signals (positions +2476 to +2480 and +2483 to +2488) were identified in the 3′ flanking region.

An intron of 870 bases long was identified in the 3′ untranslated region of this extensin gene. The canonical GT-AG rule was also applied to this intron. The intron showed an AT content of 76.2%, which is within the range of 42 to 89% in dicots (Simpson et al., 1993). However, the sequences around the splice sites did not match well with the plant consensus sequences, having an ~50% AT content compared with 72% in dicots. This type of intron located in the 3′ untranslated region seems to occur only in *HRGP* and *PRP* genes (Chen and Varner, 1985; Salts et al., 1992; Wycoff et al., 1995). The intron in the *SbHRGP3* gene is the longest of the *HRGP* and *PRP* genes reported to date.

The *SbHRGP3* gene has one open reading frame encoding a polypeptide of 432 amino acids with a molecular weight of 49,190. The polypeptide contains a putative signal peptide of 27 amino acids at the N terminus. The putative mature SbHRGP3 protein is composed of two different domains: domain 1 repeat units consist of Ser-Pro_4-Lys-His-Ser-Pro_4-Tyr_3-His, whereas domain 2 consists of Ser-Pro_4-Val-Tyr-Lys-Tyr-Lys-Ser-Pro_4-Tyr-Lys-Tyr-Pro-Ser-Pro_5-Tyr-Lys-Tyr-Pro-Ser-Pro_4-Val-Tyr-Lys-Tyr-Lys repeats (Figure 3A). Domain 1 is 176 amino acids long and is made up of 11 repeat units of 16 amino acids; domain 2 is 185 amino acids long and is made up of almost five repeat units of 39 amino acids. The repeat unit of domain 2 contains Ser-Pro_4-Val-Tyr-Lys-Tyr-Lys, which is the P2-type repeat motif of tomato extensins (Smith et al., 1986). It also contains the Tyr-Lys-Tyr-Lys block, which is known to participate in intramolecular IDT formation (Kieliszewski and Lamport, 1994).

Hydropathy plot analysis (Kyte and Doolittle, 1982) showed the distinctive repetitive structure of SbHRGP3. As shown in Figure 3B, the regions of the signal peptide, domain 1, and domain 2 could be easily determined. The entire SbHRGP3 protein is hydrophilic, except for the signal peptide. The putative cleavage site of the signal peptide is between Ala and Asp (von Heijne, 1986), which differs from that of a tobacco extensin secreted into a culture medium having a cleavage site between Asp and Asn (Kawasaki, 1991).

The putative mature polypeptide of SbHRGP3 consists of Pro/Hyp (49.0%), Tyr (19.6%), Ser (11.4%), Lys (10.6%), His (6.0%), Val (2.5%), Ile (0.5%), Ala (0.2%), and Asn (0.2%). This content is similar to those of other known extensins; however, SbHRGP3 has a slightly higher content of Pro, Tyr, and Ser residues. Due to the high content of Lys and His, SbHRGP3 is predicted to be a very basic protein with a pI of 9.9.

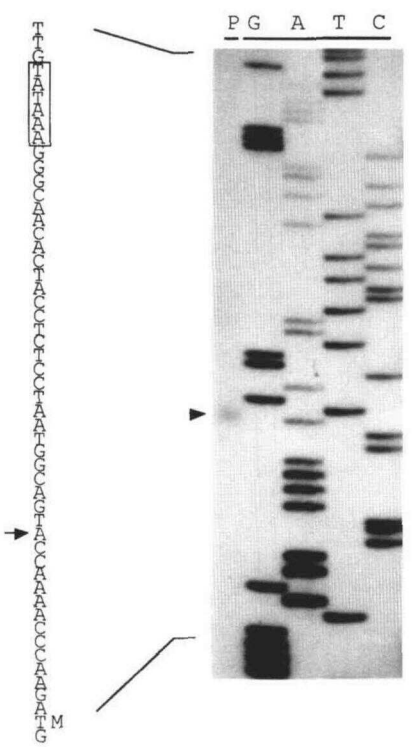

Figure 2. Primer Extension Analysis Determines the 5′ End of the *SbHRGP3* mRNA.

A 23-nucleotide primer was annealed to total RNA and extended with reverse transcriptase to yield the product in lane P indicated by an arrowhead. The same primer was used to generate the adjacent sequence ladder from a single-strand DNA template containing the *SbHRGP3* gene. The coding strand DNA sequence corresponding to the region around the extended product (positions −37 to +16 in Figure 1) is presented on the left side of the ladder. The putative TATA box is boxed, and the translation start codon (ATG) is designated by M. The putative transcription start site is indicated by an arrow.

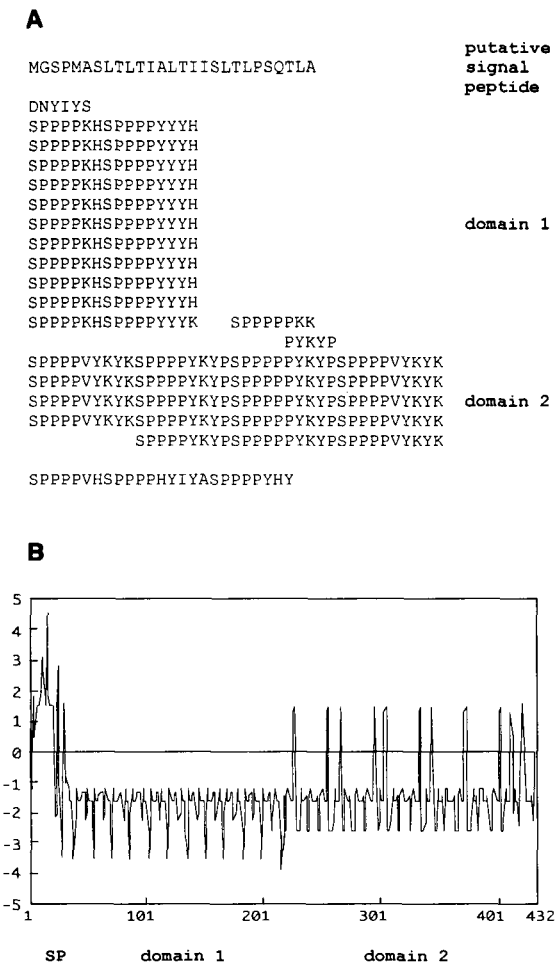

Figure 3. Deduced Amino Acid Sequence and Hydropathy Plot of SbHRGP3.

(A) Deduced amino acid sequence of SbHRGP3. The sequence was arranged to maximize its highly ordered patterns.

(B) Hydropathy plot of SbHRGP3. The hydropathy value of each amino acid was calculated by the algorithm of Kyte and Doolittle (1982) over a window of three amino acids and was plotted as a function of amino acid position. Values above the horizontal line indicate the hydrophobic regions, and values below the horizontal line indicate the hydrophilic regions. SP indicates a putative signal peptide of SbHRGP3.

Phylogenetic Status of SbHRGP3

To investigate the phylogenetic status of SbHRGP3 among dicot extensins, we found the deduced amino acid sequences of 24 dicot extensins in the GenBank data base and constructed a phylogenetic tree by using the distance matrix method (Saitou and Nei, 1987). Four graminaceous monocot extensins were also used as references.

As shown in Figure 4, three separate groups are apparent in dicot extensins with the exception of five extensins. In our analysis, groups I and III are the major groups of dicot extensins,

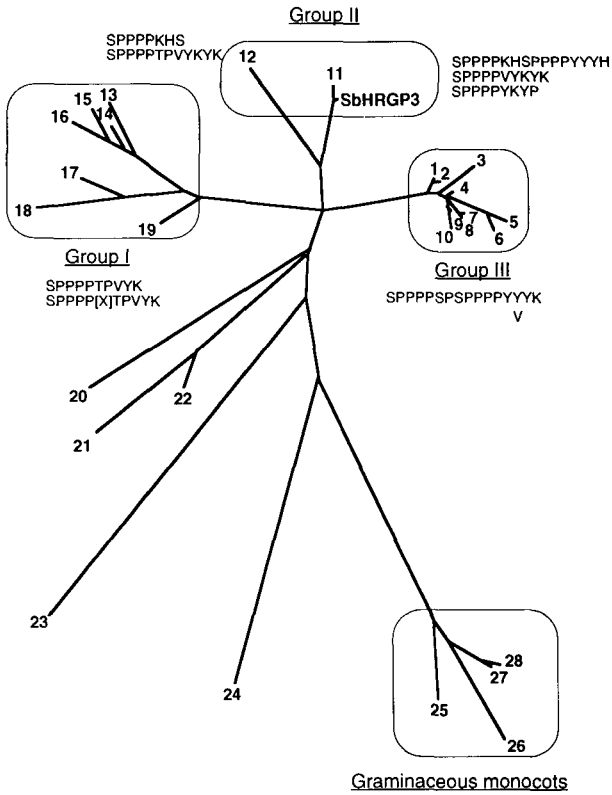

Figure 4. Phylogenetic Status of SbHRGP3 in Plant Dicot Extensins.

Amino acid sequences of 24 dicot extensins were obtained from the GenBank data base and are compared using four graminaceous monocot extensins as references. Three groups are apparent in the dicot extensins with the exception of five extensins having no apparent repeat motifs. Graminaceous monocot extensins formed a group separate from the dicot extensins. [X] in group I extensins is usually a Lys_2-Pro-Tyr_2-Pro_2-His insertion sequence. Number 1 is the cowpea extensin ext127 (accession number X86028); 2, soybean extensin SbHRGP1 (L22029; Hong et al., 1994); 3, tomato extensin (M76670; Zhou et al., 1992); 4, tomato extensin uG-18 (X55685); 5, soybean extensin SbHRGP2 (L22030; Hong et al., 1994); 6, cowpea extensin clone ext3 (X86029); 7, bean extensin (M18093; Corbin et al., 1987); 8, bean extensin (M18095; Corbin et al., 1987); 9, cowpea extensin (X86030); 10, tomato extensin w17-1 (X55681; Showalter et al., 1991); 11, bean extensin (U18791; Wycoff et al., 1995); 12, carrot extensin (X02873; Chen and Varner, 1985); 13, potato extensin (Z21937; Bown et al., 1993); 14, *Nicotiana plumbaginifolia* extensin (M34371; De Loose et al., 1991); 15, tobacco extensin ex-nt-1 (D13951); 16, tobacco extensin (X71602; Memelink et al., 1993); 17, sunflower extensin (M76546); 18, rape extensin (A18812); 19, Arabidopsis extensin (Z18787); 20, tobacco extensin HRGPnt3 (X13885; Keller and Lamb, 1989); 21, tomato extensin (M76671; Zhou et al., 1992); 22, tomato extensin (X55686); 23, Arabidopsis extensin (Z17707); 24, tomato extensin (X55687; Showalter et al., 1991); 25, sorghum extensin (X56010; Raz et al., 1991); 26, rice extensin (X61280; Caelles et al., 1992); 27, *Zea mays* extensin (X63134); 28, *Z. diploperennsis* extensin (X64173).

having consensus sequences of Ser-Pro_4-Thr-Pro-Val-Tyr-Lys, Ser-Pro_4-(X)-Thr-Pro-Val-Tyr-Lys (where [X] is usually a Lys_2-Pro-Tyr_2-Pro_2-His insertion sequence) and/or Ser-Pro_4-Ser-Pro-Ser-Pro_4-Tyr-Tyr/Val-Tyr-Lys, respectively. The distinctive feature of group I is Thr-Pro-Val-Tyr-Lys and that of group III is Tyr-rich blocks (Tyr-Tyr/Val-Tyr-Lys) after the canonical Ser-Pro_4 pentapeptide, with or without the insertion sequence. Group II, which includes SbHRGP3, is an intermediate form between groups I and III. Although extensins belonging to group I and group III contain a single domain, group II extensins contain two different domains. Among group II extensins, there are two subforms that are more similar to group I or group III extensins, respectively. SbHRGP3 and a bean extensin (Wycoff et al., 1995) have unique Val-Tyr-Lys-Tyr-Lys sequences as well as the Tyr-rich blocks, a characteristic of group III extensins. On the other hand, a carrot extensin (Chen and Varner, 1985) has Thr-Pro-Val-Tyr-Lys-Tyr-Lys, which is a characteristic of group I extensins, as well as Ser-Pro_4-Lys-His-Ser, which is a unique feature of SbHRGP3 and the bean extensin. Five other dicot extensins had no apparent repeat motifs. Four graminaceous monocot extensins used as references formed a group separate from the dicot extensins. From this analysis, we suggest that group II, including SbHRGP3 and two other extensins, could be classified as a new group of dicot extensins containing two different domains in a single polypeptide.

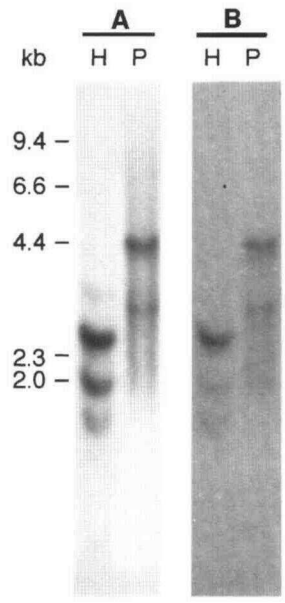

Figure 5. Genomic DNA Gel Blot Analysis.

Genomic DNA isolated from soybean leaves was digested with HindIII (H) or PstI (P) and hybridized with the coding region of the *SbHRGP3* gene after labeling by random priming. After hybridization, the filter was washed under low- or high-stringency conditions.
(A) Filter washed under low-stringency conditions.
(B) Filter washed under high-stringency conditions.
Numbers at left indicate length markers in kilobases.

The *SbHRGP3* Gene Is a Member of the Extensin Gene Family in Soybean

To determine the copy number of extensin genes in the soybean genome, we performed a DNA gel blot analysis. As shown in Figure 5A, two or three positive signals are visible in each lane. When the filter was washed under more stringent conditions, a single prominent band appeared in each lane (Figure 5B). This indicates that soybean extensins are encoded by a relatively small number of genes. However, the *SbHRGP3* gene probably exists as a single copy in the genome.

Expression of the *SbHRGP3* Gene in Soybean Seedlings Is Developmentally Regulated

To investigate the stage-specific expression of the *SbHRGP3* gene in soybean seedlings, we extracted RNA from hypocotyls of 1-, 3-, 5-, and 7-day-old seedlings and hybridized the RNA with a gene-specific probe. Figure 6A shows the pattern of *SbHRGP3* mRNA accumulation in developing hypocotyls. The mRNA level increased approximately threefold at ~5 days after germination and decreased in 7-day-old seedlings.

To examine the spatial distribution pattern of *SbHRGP3* mRNA in seedlings, we serially dissected the 5-day-old seedlings, as shown in Figure 6B. RNAs from each section were hybridized with the gene-specific probe. As shown in Figure 6C, the *SbHRGP3* gene was expressed highly in the mature region of the hypocotyl when compared with apical and elongating regions of the plant. Expression levels among different sections of the mature region were similar but still three- to sixfold higher than in apical and elongating regions. The expression level of the *SbHRGP3* gene in the root was similar to the level in the mature region of the hypocotyl.

Expression of the *SbHRGP3* Gene in Transgenic Tobacco Plants

Recently, transgenic plants carrying extensin promoters driving β-glucuronidase (*GUS*) expression have been used to illustrate both tissue- and gene-specific expression of extensin genes (Showalter, 1993; Wycoff et al., 1995). The 5′ flanking region of the *SbHRGP3* gene was amplified by polymerase chain reaction, and the product was fused with the *GUS* reporter gene. The *SbHRGP3–GUS* chimeric gene was introduced into tobacco, and its expression was examined in various tissues. Transgenic plants transformed with the pGA643 vector (An et al., 1988) were used as a control.

Histochemical GUS assays showed that the *SbHRGP3–GUS* construct was not induced by wounding in leaves of transgenic tobacco plants (Figure 7A), even though there are many reports that expression of extensin genes is wound inducible (Memelink et al., 1993; Parmentier et al., 1995; Wycoff et al.,

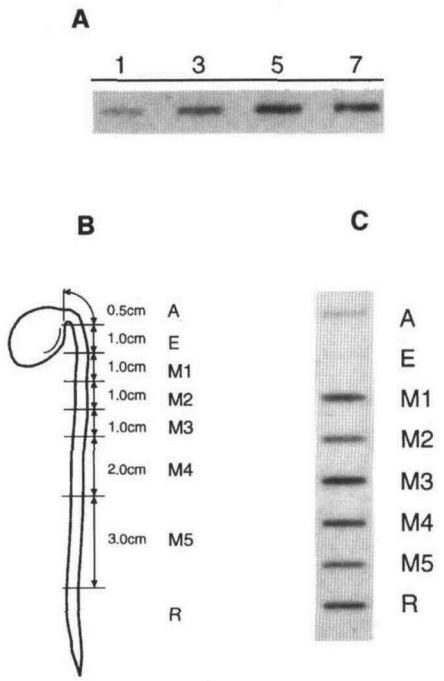

Figure 6. Expression Pattern of the *SbHRGP3* Gene in Soybean Seedlings.

(A) RNA slot blot showing stage-specific expression of the *SbHRGP3* gene in 1-, 3-, 5-, and 7-day-old soybean seedlings. The coding region of the *SbHRGP3* gene was used as a probe.

(B) Serial dissection of 5-day-old seedlings. Five-day-old seedlings were dissected into hypocotyls and roots (R). Hypocotyls were further dissected into apical (A), elongating (E), and five mature sections (M1 to M5).

(C) RNA slot blot showing spatial distribution of *SbHRGP3* mRNA in hypocotyls and roots. Five micrograms of total RNA isolated from each sample in **(B)** was applied to a nylon membrane and hybridized with the random primer labeled probe.

1995). However, GUS activity was observed in wounded leaves when they were floated on medium supplemented with 3% sucrose (Figure 7B). Further investigation showed that *GUS* expression was not induced in leaves that were not wounded, even when they were floated on medium supplemented with sucrose (Figure 7C). A cross-section of the wounded leaves showed that *GUS* expression is localized in phloem tissues and cambium cells (Figure 7D). The *SbHRGP3–GUS* gene is expressed similarly in phloem tissues of petioles (Figures 7E and 7F). Light or dark conditions did not affect the tissue-specific expression of the *SbHRGP3–GUS* gene in the transgenic tobacco plants (data not shown). The control tissues transformed with pGA643 did not show GUS activity under any of the conditions tested (data not shown).

The chimeric gene was not induced in stems by wounding alone (Figure 8A); however, the chimeric gene was induced in the presence of sucrose as occurred in leaves (Figure 8B). The expression of the *SbHRGP3–GUS* gene is specific to inner and outer phloem cells (Figures 8C and 8D). However, the chimeric gene was not induced in roots by wounding in the absence or presence of sucrose (data not shown).

To assess the effect of ethylene in the induction of the *SbHRGP3–GUS* gene, we floated unwounded and wounded tissues on a medium containing 0.5 mg/mL ethephon (2-chloroethylphosphonic acid). All tested tissues, including leaves, stems, and roots, did not express GUS activity when treated with ethephon (data not shown).

Because both wounding and sucrose are required for the expression of the *SbHRGP3–GUS* gene, we examined the concentration of sucrose required for the maximum activity. Leaves of transgenic tobacco plants were wounded and floated on medium supplemented with various concentrations of sucrose. After floating for 24 hr, the tissues were homogenized and a fluorometric assay was performed to quantitate GUS activity. As shown in Figure 9, wounded leaves floated on sucrose-free medium and unwounded leaves showed no GUS activity. On the other hand, all wounded leaves that floated on medium containing sucrose showed GUS activity, reaching the maximum at a 9% sucrose concentration.

Expression of the *SbHRGP3* Gene in Transgenic Tobacco Seedlings

Transgenic tobacco plants were self-pollinated, and seed were harvested. Seed were germinated in the light for various time periods and histochemically assayed for GUS activity. As shown in Figure 10, the *SbHRGP3–GUS* gene is expressed in a distinct pattern during seedling development. GUS activity was not detected in the seedlings at the early stage of germination (Figures 10A and 10B). However, GUS activity was observed in the mature regions of the hypocotyl and root of the 3-day-old seedling (Figure 10C). GUS activity was not observed in 4- and 5-day-old seedlings (Figures 10D and 10E). GUS activity was once again detected in a specific zone of the root 6 to 8 days after germination (Figures 10F and 10G). However, GUS activity was not detected in this zone in the 10-day-old seedling (Figure 10H). Although GUS activity was not observed in the 12-day-old seedling, which was developing a lateral root (Figure 10I), it was observed again in the lateral root of the 14-day-old seedling (Figure 10J). GUS activity was also detected inside of the seed coat of the 3-day-old seedling (Figure 10K). Cross-sections of roots of the 3- and 8-day-old seedlings indicated that GUS activity is restricted to a layer of cells in the epidermis (Figures 10L and 10M).

Because GUS activity could once again be detected in a specific zone in the roots of the 6- and 8-day-old seedlings (Figures 10F and 10G), we closely inspected seedlings at sequential stages during the initiation and development of lateral roots. As shown in Figure 11A, the zone with GUS activity is a zone from which a lateral root is to be initiated. As a subset of pericycle and endodermal cells started to divide to initiate the lateral root, GUS activity slowly decayed in this zone (Figures 11B to 11D). When the lateral root emerged from the primary root, no GUS activity was observed (Figures 11E and 11F). Moreover, GUS activity was not detected at the tip of the

developing lateral root, which is not the case for extensin genes of tobacco (Keller and Lamb, 1989) and bean (Wycoff et al., 1995).

These results indicate that the *SbHRGP3* gene is differentially regulated in various regions of the seedlings at different developmental stages. The *SbHRGP3* gene may play an important role in maturation and cell wall reformation of primary and lateral roots.

DISCUSSION

We have isolated and characterized a novel soybean extensin gene. The *SbHRGP3* gene encodes a putative HRGP having two domains with different repeat motifs. The *SbHRGP3* gene is expressed in the mature region of hypocotyls and in roots of soybean seedlings. Wounding did not induce the expression of the *SbHRGP3–GUS* gene in either leaves or stems of transgenic tobacco plants, although there are many reports that expression of extensin genes is wound inducible (Memelink et al., 1993; Showalter, 1993; Wycoff et al., 1995). However, we found that wound induction of the *SbHRGP3–GUS* gene absolutely requires sucrose. Expression was specific to phloem tissues and cambium cells in leaves and stems. Furthermore, its expression was modulated in a distinct pattern during transgenic seedling development.

SbHRGP3 Has a Unique Structure with Two Different Domains

SbHRGP3 has two domains, each having different motifs, as shown in Figure 3. Domain 1 is composed of 11 repeat units

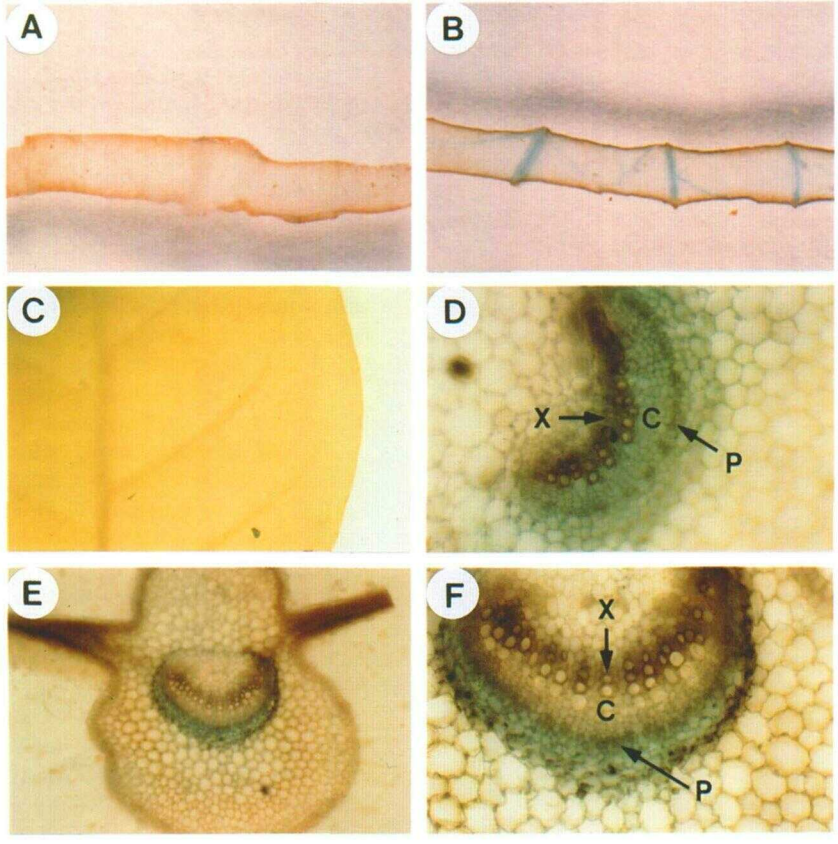

Figure 7. Histochemical Localization of GUS Activity in Leaves of Transgenic Tobacco Plants.

(A) Leaf showing that expression of the *SbHRGP3–GUS* gene was not induced by wounding alone.
(B) Leaf showing that expression of the *SbHRGP3–GUS* gene was induced by wounding in the presence of 3% sucrose.
(C) Leaf showing that expression of the *SbHRGP3–GUS* gene was not induced by floating unwounded leaves on medium with 3% sucrose.
(D) Cross-section of the leaf shown in **(B)** in which expression of the *SbHRGP3–GUS* gene was induced by wounding in the presence of sucrose.
(E) Cross-section of a petiole in which expression of the *SbHRGP3–GUS* gene was induced by wounding in the presence of sucrose.
(F) Magnification of the petiole shown in **(E)**.
C, cambium; P, phloem; X, xylem.

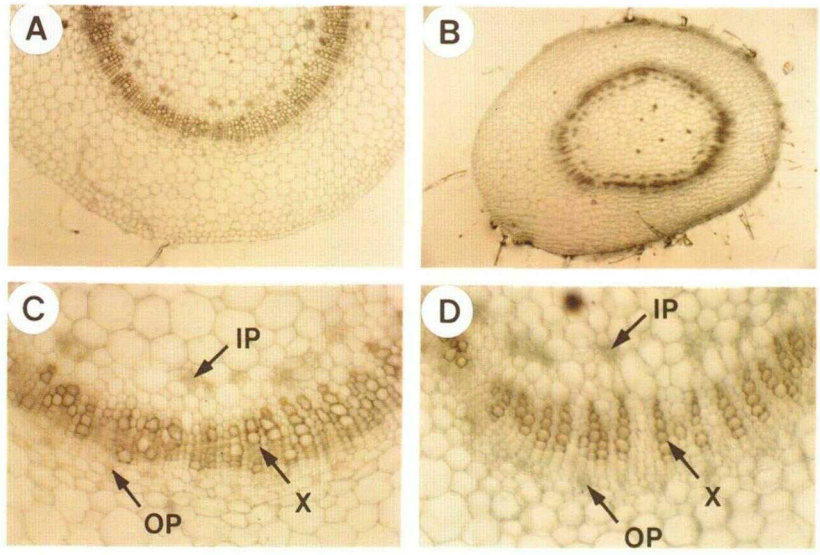

Figure 8. Histochemical Localization of GUS Activity in Stems of Transgenic Tobacco Plants.

(A) Cross-section of a stem in which expression of the *SbHRGP3–GUS* gene was not induced by wounding alone.
(B) Cross-section of a stem in which expression of the *SbHRGP3–GUS* gene was induced by wounding in the presence of 3% sucrose.
(C) and **(D)** Magnification of the sections shown in **(A)** and **(B)**, respectively. IP, inner phloem; OP, outer phloem; X, xylem.

(Ser-Pro_4-Lys-His-Ser-Pro_4-Tyr_3-His) containing the Tyr-Tyr-Tyr-His block, whereas domain 2 has five repeat units (Ser-Pro_4-Val-Tyr-Lys-Tyr-Lys-Ser-Pro_4-Tyr-Lys-Tyr-Pro-Ser-Pro_5-Tyr-Lys-Tyr-Pro-Ser-Pro_4-Val-Tyr-Lys-Tyr-Lys) containing the Val-Tyr-Lys-Tyr-Lys block. The two blocks may be involved in intramolecular and intermolecular IDT cross-linking (Kieliszewski and Lamport, 1994). The repeat units of domain 2 also contain unusual repetitive sequences (Tyr-Lys-Tyr-Pro) that may also participate in IDT bridge formation. The presence of these putative functional sites suggests that SbHRGP3 could be classified as an intermolecular and intramolecular cross-linking extensin (Kieliszewski and Lamport, 1994). An extensin gene of bean (Wycoff et al., 1995) was recently reported to encode an HRGP with a structure similar but not identical to that of SbHRGP3.

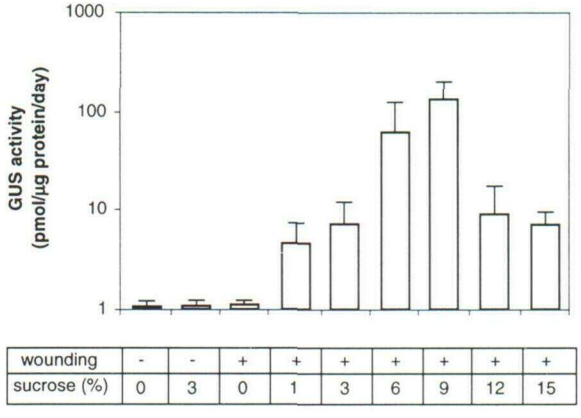

Figure 9. Fluorometric Assay for GUS Activity in Leaves of Transgenic Tobacco Plants Incubated in Medium Containing Various Concentrations of Sucrose.

Unwounded (–) or wounded (+) leaf sections of transgenic plants were incubated for 24 hr in medium with or without sucrose at various concentrations, as indicated. Thirty micrograms of protein extracted from each sample was assayed fluorometrically for GUS activity. The error bars indicate the standard deviation for the three replicate experiments in each set.

In most cases, a single extensin polypeptide contains a single-type repeat motif with either a Tyr-X-Tyr-Lys block (Corbin et al., 1987; Showalter et al., 1991; Zhou et al., 1992) or a Thr-Pro-Val-Tyr-Lys block (Memelink et al., 1993). However, SbHRGP3 contains both Tyr-Tyr-Tyr-His and Val-Tyr-Lys-Tyr-Lys blocks. Moreover, the structure of SbHRGP3 is novel in that it contains two different domains.

Another novel feature of SbHRGP3 is that it contains the repetitive Ser-Pro_5 hexapeptide block. Only a few extensins with Ser-Pro_5 blocks have been reported (Zhou et al., 1992). Although most dicot extensins contain Ser-Pro_4 repeats, some extensins from primitive species contain more than four Pro residues after Ser and have irregular repetitive structures (Ertl et al., 1992). Whether SbHRGP3 is a more ancient form of other canonical extensins or a chimeric extensin evolved recently from two extensins with different repeat motifs (Kieliszewski and Lamport, 1994; Wycoff et al., 1995) remains to be elucidated.

Domain 1 of SbHRGP3 has motifs similar to P3-type extensins (Ser-Pro_4-Ser-Pro-Ser-Pro_4-Tyr_3-Lys). Although the Tyr-rich blocks have been reported in some dicot extensins (Showalter, 1993), the repeat unit is somewhat different from that of domain 1. The repeat unit in domain 1 of SbHRGP3 has the Tyr-rich block with His instead of Lys residues, and Ser-Pro_4-

Lys-His-Ser instead of the palindromic Ser-Pro_4-Ser-Pro-Ser-Pro_4 sequence. The repeat unit (Ser-Pro_4-Lys-His-Ser-Pro_4-Tyr_3-His) has only been reported for SbHRGP3 and a bean extensin (Wycoff et al., 1995). It has only been suggested that His residues in some cell wall proteins (Sheng et al., 1991; Kieliszewski et al., 1992) might be involved in a metal binding function mediating intramolecular and intermolecular protein–protein interactions (van Dam et al., 1989) or interactions between uronic acid components of other cell wall polymers as part of a self-assembly process (Kieliszewski and Lamport, 1994). Therefore, it is possible that the His residues of SbHRGP3 may serve as ionic interaction sites for protein–carbohydrate or protein–protein interactions in cell wall formation.

Hong et al. (1994) reported on two other partial extensin cDNAs expressed in soybean hypocotyls, *SbHRGP1* and *SbHRGP2*. Their transcripts are 5.4 and 3.0 kb long, respectively. SbHRGP1 contains Ser-Pro_4-Ser-Pro_4-Tyr-Val-Tyr-Lys motifs, whereas SbHRGP2 contains Ser-Pro_4-Ser-Pro_4-Tyr_3-His/Lys motifs. Although all of the three extensins in soybean have the consensus sequence of Tyr-rich blocks (Tyr-X-Tyr-His/Lys, where X is most frequently Tyr), SbHRGP3 has a more strict repeat pattern than the other two soybean extensins. It is conceivable that the differences in their repeat motifs might drive them to exhibit different properties in cell wall formation (Kieliszewski and Lamport, 1994).

SbHRGP3 Belongs to a New Group of Extensins

Based on the phylogenetic tree of the repetitive motifs (Figure 3), we suggest that SbHRGP3 and two other extensins of carrot and bean (Chen and Varner, 1985; Wycoff et al., 1995) may form a new group in dicot extensins. Group I in our tree includes the P1-type extensins from tomato, sycamore, tobacco, petunia, and carrot (Lamport, 1977; Chen and Varner, 1985; Smith et al., 1986; Showalter, 1993), and group III includes the tomato P3-type cell wall extensins (Epstein and Lamport, 1984; Smith et al., 1986). SbHRGP3 has two motifs similar to the P2- and P3-type extensins, respectively, as has been observed in the bean extensin (Wycoff et al., 1995). Recently, Kieliszewski and Lamport (1994) reported on the putative phylogeny of the extensin HRGP family. They dichotomized the extensin HRGP family based on whether their sequences contained Val-Tyr-Lys or not. SbHRGP3 contains both motifs with and without Val-Tyr-Lys, respectively, suggesting that SbHRGP3 is structurally intermediate between the two groups.

Sucrose Requirement for Wound-Inducible Expression of the *SbHRGP3* Gene in Leaves and Stems of Transgenic Tobacco Plants

Almost all extensin genes have been reported to be induced by wounding (Corbin et al., 1987; Templeton et al., 1990; Showalter, 1993; Wycoff et al., 1995). Ecker and Davis (1987) previously showed that extensin transcripts were induced by ethylene in carrot roots. Furthermore, Memelink et al. (1993) showed that the wound-inducible expression of an extensin gene in tobacco was suppressed by $CoCl_2$, an inhibitor of ethylene formation (Yang and Hoffman, 1984). All of these data imply that extensin genes are prevalently induced by wounding. However, the expression of the *SbHRGP3–GUS* gene was not induced by either wounding or ethephon treatment alone but by wounding in the presence of sucrose (Figures 7 and 8). Its expression is specific to phloem tissues and cambium cells. The maximum level of the wound-inducible *SbHRGP3–GUS* gene expression in leaves of the transgenic tobacco plants was observed at 9% sucrose (Figure 9). This suggests that an adequate level of sucrose may not be available in tobacco leaves to drive the wound-inducible *SbHRGP3–GUS* gene expression.

It was reported previously that wounding in combination with sucrose strongly induced a proteinase inhibitor II gene in transgenic tobacco (S.-R. Kim et al., 1991). However, the gene could be induced to a considerable amount by wounding alone. S.-R. Kim et al. (1992) showed that a 23-mer sequence in the 5′ flanking region of the potato proteinase inhibitor II gene could restore the sucrose response as well as the wound response. The *SbHRGP3* gene contains a sequence (GCTTGCT) at approximately position −410 that is included in the 23-mer sequence of the proteinase inhibitor II gene. The role of the putative regulatory sequence in sucrose induction of the *SbHRGP3* gene requires further investigation.

We do not know why wound induction of the *SbHRGP3* gene absolutely requires sucrose. It is unlikely that sucrose is required for wound induction of *SbHRGP3* expression simply for providing energy to the phloem tissues and cambium cells where the *SbHRGP3* gene was specifically induced by wounding. Rather, it may act as a specific signal in the wound signal transduction pathway, as suggested by S.-R. Kim et al. (1991). Sucrose may mediate the wound signal transduction in the phloem tissues and cambium cells by binding to a specific receptor(s) in their cell membranes.

The wound inducibility and tissue specificity of the *SbHRGP3* gene suggest that SbHRGP3 may not be involved in plant defense simply by helping to heal wounds or by forming a physical barrier against invading pathogens. Rather, it may function by increasing the structural reinforcement of wounded tissues, thereby preventing pathogens from entering into the vascular system, which otherwise leads to pathogen spreading to other regions of the plant.

Temporal Activation of the *SbHRGP3* Gene during Seedling Development

The *SbHRGP3–GUS* gene is expressed differentially during transgenic seedling development (Figure 10). The *SbHRGP3–GUS* gene was not expressed in the seedlings during the early stages of germination (Figures 10A and 10B). However, it was induced later in the mature regions of hypocotyls and roots

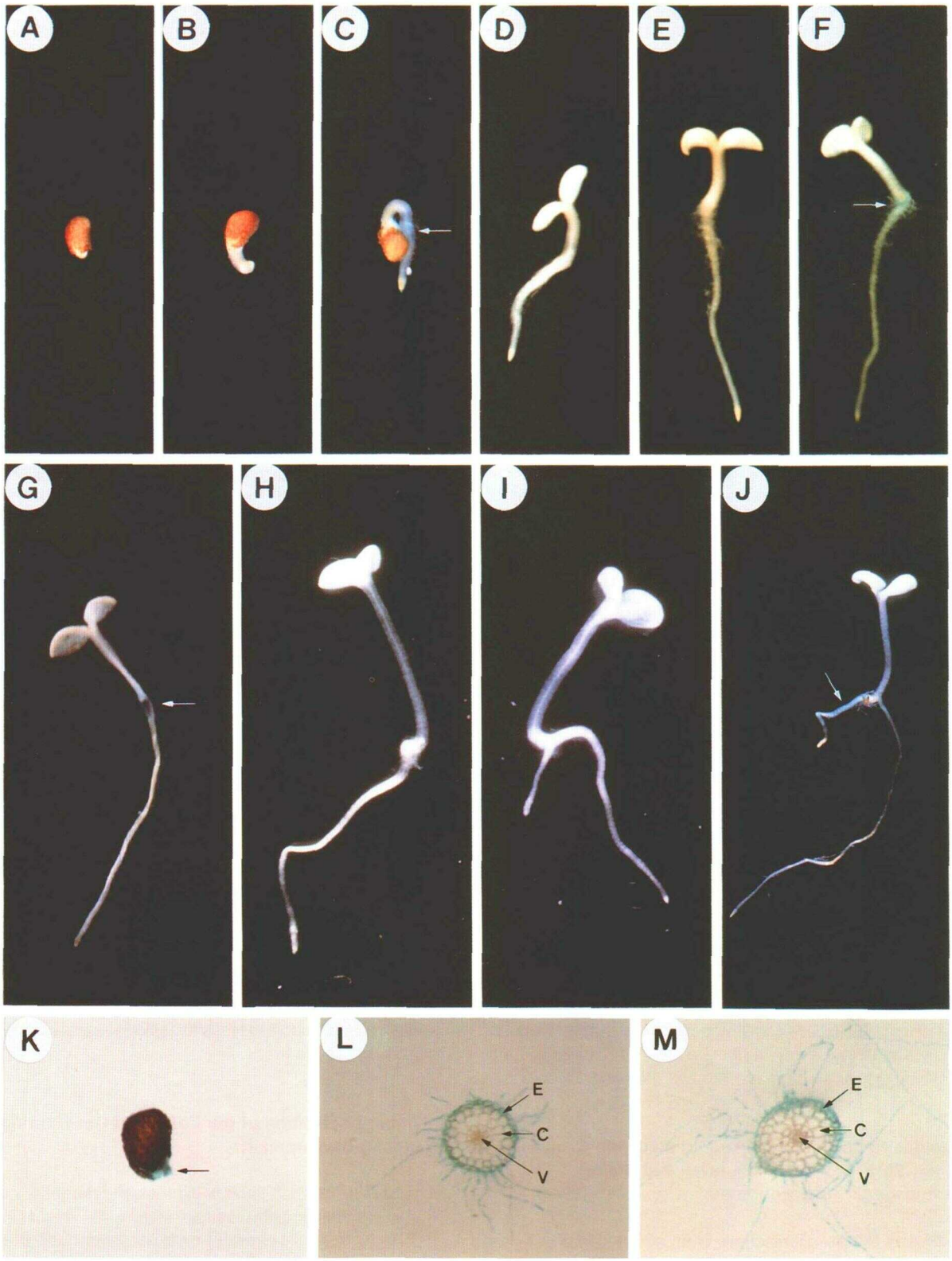

Figure 10. Histochemical Localization of GUS Activity in Germinating Seedlings of Transgenic Tobacco Plants.

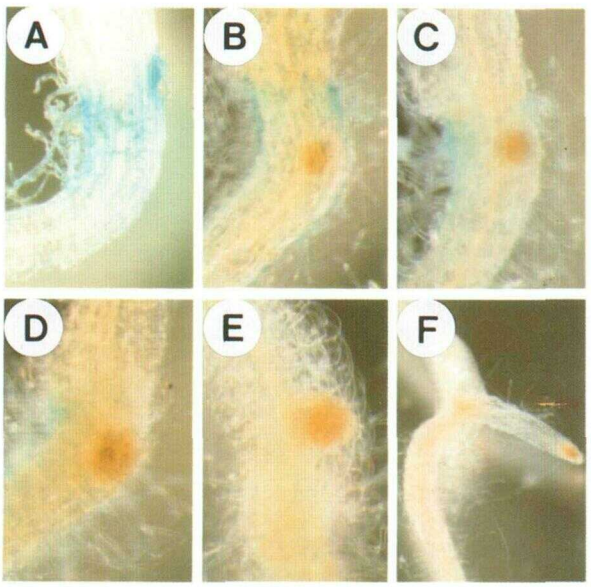

Figure 11. Histochemical Localization of GUS Activity in Transgenic Seedlings at Sequential Stages of Lateral Root Initiation and Development.

The region from which a lateral root is being initiated from the primary root of the transgenic seedling is shown close up.
(A) Primary root of an 8-day-old seedling showing GUS activity in a specific zone, as shown in Figure 10G.
(B) to **(E)** Primary roots from which lateral roots are being initiated.
(F) Primary and lateral roots.

of transgenic seedlings (Figure 10C). The *SbHRGP3–GUS* gene was also induced at the mature part of lateral roots (Figure 10J). This indicates that SbHRGP3 may play a role in hypocotyl and root maturation.

In addition, the *SbHRGP3–GUS* gene was reactivated at a specific zone in the primary root of transgenic seedlings (Figures 10F and 10G). Expression was localized at a layer of the epidermal cells of the zone (Figure 10M), from which a lateral root was to be initiated (Figure 11).

This distinct pattern of the *SbHRGP3* gene expression may provide information on the genetic program of lateral root formation (Dolan et al., 1993). In angiosperm roots, derivatives of both the pericycle and endodermis contribute to the new root primordium, which later must break through the cortex and epidermis of the parental root. Therefore, SbHRGP3 seems to play a role in cell wall reformation at the epidermis, which may be required for initiation and development of the lateral root from the parental root.

Keller and Lamb (1989) reported on a novel *HRGP* gene of tobacco that was expressed in a cell type–specific manner during lateral root initiation. Tobacco *HRGP* expression was specific to a subset of the pericycle and endodermal cells from which a lateral root originated. Its expression continued in the cells at the tip of the emerging lateral root. However, *SbHRGP3* expression is specific to the epidermal cells of the zone from which the lateral root is to be initiated but was inactivated when the pericycle and endodermal cells began to divide. The tobacco HRGP is considered to play a role in the hardening of cell walls of the root tip, thereby giving the root tip the mechanical strength necessary for penetrating through the cortex and epidermis of the main root (Keller and Lamb, 1989). In contrast, SbHRGP3 may be involved in the hardening of the cell walls in the epidermis, which the lateral root must break through. This indicates that SbHRGP3 may have a specialized structural function, possibly in reducing any severe damage to the cells of the epidermis and/or the cortex caused by the lateral root breaking through. Therefore, the *SbHRGP3* gene exemplifies the selective activation of extensin genes in specific cells for precise morphogenetic control of cell wall architecture during root differentiation.

Because the *SbHRGP3* gene is not only expressed in a tissue-specific manner in response to both wounding and sucrose but is also regulated developmentally in hypocotyls and roots, further studies are needed to determine the information required for the expression at different tissues to different regulatory stimuli.

METHODS

Plant Materials

Soybean (*Glycine max* cv Paldal) and tobacco (*Nicotiana tabacum* cv Xanthi) were used for all experiments. The plants were grown at 28°C under a 16-hr-light/8-hr-dark cycle. The hypocotyls of 6-day-old soybean seedlings grown in the dark were used to isolate total RNA for primer extension analysis.

For stage-specific expression of the soybean hydroxyproline-rich glycoprotein (*SbHRGP3*) gene, hypocotyls of 1-, 3-, 5-, and 7-day-old

Figure 10. (continued).

Seeds obtained from self-pollinated transgenic tobacco plants were germinated in Petri dishes for various periods and assayed for GUS activity.
(A) to **(G)** One-, 2-, 3-, 4-, 5-, 6-, and 8-day-old seedlings, respectively.
(H) and **(I)** Ten- and 12-day-old seedlings, respectively. Shown is a lateral root emerging in the 12-day-old seedling shown in **(I)**.
(J) Fourteen-day-old seedling.
(K) Seed coat of the 3-day-old seedling in **(C)**.
(L) Cross-section of the seedling shown in **(C)**.
(M) Cross-section of the seedling shown in **(G)**.
Arrows in **(C)**, **(F)**, **(G)**, **(J)**, and **(K)** indicate zones where GUS activity was detected. C, cortex; E, epidermis; V, vascular cylinder.

seedlings were used. For spatial distribution of *SbHRGP3* mRNA, the 5-day-old seedlings were dissected into hypocotyl and root. Hypocotyl was further sectioned into apical, elongating, and five mature regions, as described previously by Hong et al. (1989).

Seeds of transgenic tobacco plants (R_0) were obtained by self-pollination, and the R_1 progeny were aseptically germinated on wet filter paper in Petri dishes under a 16-hr-light/8-hr-dark cycle.

Isolation of Nucleic Acids

Genomic DNA and total RNA were isolated according to standard methods (Ausubel et al., 1987; Chomczynski and Sacchi, 1987).

Screening of a Genomic Library

A soybean genomic library constructed in EMBL3 (C.H. Kim et al., 1992) was screened with a cDNA clone, SE126, which encodes an extensin in soybean (Choi, 1995), as described by Sambrook et al. (1980). Labeling by random priming was with the Prime-a-Gene system (Promega). A 14.0-kb SalI fragment containing the *SbHRGP3* gene was subcloned into pUC19 by using standard procedures (Sambrook et al., 1989).

DNA Sequencing and Phylogenetic Analysis

The nucleotide sequence of the *SbHRGP3* gene was determined by the dideoxynucleotide chain termination method. Both strands of the coding region containing the repetitive sequence structures were sequenced with Taq DNA polymerase.

The nucleotide sequence and deduced amino acid sequences were analyzed with the Macintosh DNASIS program (Hitachi Software Engineering America, Ltd., San Bruno, CA). Multiple sequence alignments of extensins obtained from the GenBank data base were performed by using the Clustal V program (Higgins et al., 1992), and a phylogenetic tree was constructed by using the distance matrix method (Saitou and Nei, 1987), with no outgroup being selected.

Primer Extension Analysis

RNA isolated from hypocotyls was hybridized with a synthetic oligonucleotide (5′-TGTCTGCTAATGTTTGAGATGGC-3′) from positions +76 to +98 of the *SbHRGP3* gene. The synthesized first-strand cDNA was electrophoresed in a 6% denaturing polyacrylamide gel (Sambrook et al., 1989). The oligonucleotide was also used as a primer to sequence a DNA fragment containing the *SbHRGP3* gene for size markers.

DNA Gel Blot Hybridization

Ten micrograms of genomic DNA was digested with restriction enzymes and electrophoresed in a 0.7% agarose gel. The DNA was transferred onto a nylon membrane, as described earlier (Sambrook et al., 1989). The membrane was hybridized with a random primer labeled probe corresponding to the coding region of the *SbHRGP3* gene. The coding region of the *SbHRGP3* gene was amplified by polymerase chain reaction, using the EXTP5 primer (5′-GGCAGTACCAAAACCCAAGATGGGGTCTC-3′ from positions −6 to +23) and EXTP6 primer (5′-TGAAGAAGCTTACTACTCGGGAAGCTAAG-3′ from positions +1325 to +1353). The membrane was washed at 65°C with 1 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate) under low-stringency conditions or with 0.1 × SSC under high-stringency conditions.

RNA Slot Blot Hybridization

Five micrograms of total RNA from various sections of soybean seedlings was denatured and transferred onto a nylon membrane by using a manifold (Bio-Rad). Five micrograms of total RNA from hypocotyls of 1-, 3-, 5-, and 7-day-old seedlings was also processed as given above. The membrane was hybridized with the random primer labeled probe in a solution of 50% formamide, 5 × SSPE (1 × SSPE is 0.15 M NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 7.4), 5 × Denhardt's solution (1 × Denhardt's solution is 0.02% Ficoll, 0.02% PVP, 0.02% BSA), 0.1 mg/mL sheared salmon sperm DNA, and 0.5% SDS at 42°C overnight. The membrane was washed under high-stringency conditions at 70°C. For quantitation, the membrane was analyzed with the BAS-1000 PhosphorImager (Fuji Photo Film Co., Tokyo).

Chimeric Gene Construction and Plant Transformation

The 5′ flanking region (0.9 kb) of the *SbHRGP3* gene was amplified by polymerase chain reaction in Thermal Cycler-1 (Perkin-Elmer) by using the EXTP2 primer (5′-GTGTGCATAAGCTTTCCACATGTCAC-3′ from positions −922 to −897) and EXTP3 primer (5′-TACGGATCCTAGGAGAGGTAGTGTTGCCC-3′ from positions −28 to +1) with HindIII and BamHI restriction sites. The polymerase chain reaction–amplified product was digested with HindIII and BamHI and fused to the DNA fragment containing the β-glucuronidase (*GUS*) reporter gene and the nopaline synthase terminator in the correct reading frame. The chimeric gene was subsequently cloned into the plant expression vector pGA482 (An et al., 1988) and introduced into tobacco cells via *Agrobacterium tumefaciens*–mediated leaf disc transformation (Horsch et al., 1985). Transformed shoots were selected on Murashige and Skoog basal medium (Gibco BRL) supplemented with 200 mg/L kanamycin and 500 mg/L carbenicillin.

Induction of Expression in Transgenic Plants

Various tissues of transgenic plants were surface sterilized with commercial bleach solution and cut into 3- to 5-mm slices with a razor blade. They were floated on liquid Murashige and Skoog basal medium with or without 3% sucrose for 24 hr. The leaf slices were also floated on the medium supplemented with 1 mg/mL $CoCl_2$ and 0.5 mg/mL ethephon (2-chloroethylphosphonic acid). Ethephon treatment caused browning of leaf slices.

Histochemical GUS Assay and Microscopy

Histochemical GUS assays were done by the standard method (Jefferson and Wilson, 1991) with some modifications. The samples were soaked in 1 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide, 50 mM sodium phosphate, pH 7.0, and 0.1% Triton X-100 for 24 hr. The samples were cleared in absolute ethanol. Indigo dye–developed samples were sectioned by hand using a double-edged razor blade.

Photographs were taken in the Nikon SMZ 10A dissection microscope (Nikon Co., Tokyo) with Kodak Ektachrome 64 daylight film.

Fluorometric Assay of GUS Activity

Transgenic plants carrying the chimeric gene were wounded as described above and floated on medium supplemented with 1, 3, 6, 9, 12, or 15% sucrose for 24 hr. Unwounded leaves floated on medium with or without 3% sucrose and wounded leaves floated on medium without sucrose were used as a control. The leaves were homogenized in extraction buffer (50 mM sodium phosphate, pH 7.0, 5 mM DTT, 1 mM EDTA, 0.1% [w/v] sarcosyl, 0.1% Triton X-100). Thirty micrograms of protein from each sample was assayed as described previously (Jefferson and Wilson, 1991). 4-Methylumbelliferyl β-D-glucuronide (1 mM) was used as the fluorogenic substrate. Excitation of the sample was performed at 365 nm, and emissions were detected at 455 nm. Freshly prepared 100 nM 4-methylumbelliferone and 1 μM 4-methylumbelliferone were used as the standards.

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