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Vascular expression of a bean cell wall glycine-rich protein - β-glucuronidase gene fusion in transgenic tobacco

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In French bean (*Phaseolus vulgaris* L.), the glycine-rich wall protein GRP 1.8 is specifically synthesized in protoxylem tracheary elements of the vascular system. A 494 bp upstream promoter fragment of the gene encoding GRP 1.8 was isolated and translationally fused to the β-glucuronidase reporter gene. Transgenic tobacco plants containing this construct expressed the gene in vascular tissue of roots, stems, leaves and flowers. The gene was developmentally expressed during differentiation of both primary and secondary vascular tissue and was also rapidly induced (in <30 min) after excision-wounding of young stems. This wound response is more rapid than in bean hypocotyls, indicating possible differences between the activation mechanism for glycine-rich protein gene expression in wounded bean and tobacco. Only a subset of cells were found to participate in the wound response. In young stems, the GRP wound induction was localized in pith parenchyma cells adjacent to the wound surface, where vessel regeneration is known to occur. Thus, a promoter fragment of 494 bp, including 427 bp upstream from the transcription start site, contains information for tissue-specific and wound-induced gene regulation. The cell-type specificity of expression suggests that the GRP 1.8 promoter is regulated by very specific developmental and environmental signals.

Key words: β-glucuronidase/cell wall/glycine-rich protein/*Phaseolus vulgaris*/xylem

Introduction

Glycine-rich proteins (GRPs) are a class of plant cell wall structural proteins which have a very repetitive primary structure, containing ~60% glycine which is predominantly arranged in $(Gly\text{-}X)_n$ repeats. A bean (*Phaseolus vulgaris* L.) GRP (GRP 1.8) was shown to be localized in a cell wall fraction using antibodies (Keller *et al.*, 1988). Two other such proteins contain hydrophobic signal sequences and are therefore expected to be cell wall proteins also (Condit and Meagher, 1986; Keller *et al.*, 1988). GRP 1.8 is associated with the vascular tissue, specifically in protoxylem tracheary elements (Keller *et al.*, 1989). The protein is co-localized with the annular and helical secondary thickenings in the cell walls of these elements, indicating a close relationship between GRP and lignin during development. In metaxylem tracheary elements of bean, no GRP was detected in the strongly extensible protoxylem elements. The GRP 1.8 gene is developmentally regulated, being strongly expressed in developing young tissue and switched off in older tissue. GRP 1.8 is wound-induced in older plants. Induction was observed 1.5 h after wounding, maximum levels were attained after 8 h. No wound-induction was observed in young tissue which is already strongly expressing the gene.

The accumulation of GRP 1.8 in a small set of cells provides a defined system for the analysis of the molecular basis of cell-type specificity in relation to the differentiation of tracheary elements in the plant vascular sysem. Many cell types retain the potential to dedifferentiate and ultimately to regenerate a new plant. However, those cells determined to become tracheary elements are programmed to die and then form the water-conducting system. No genes specifically expressed in the tracheary elements have been analyzed to date and hence little is known about the molecular mechanism underlying this differentiation pathway. The observed developmental regulation and wound-induction of the GRP 1.8 gene also provides an opportunity to study the relationship between developmental and environmental induction of a plant gene. In particular, analysis of GRP 1.8 expression at the cellular level will reveal whether the same cell-type is involved in developmental and wound-induced expression and will also give some insight into the interplay between these two regulatory networks.

Transgenic plants carrying promoter-reporter gene fusions have been used successfully for the analysis of the expression of several genes, including light- and stress-regulated genes (Schell, 1987; Willmitzer, 1988). These studies have revealed *cis*-acting regulatory elements important for various aspects of gene expression. They also demonstrated that in most cases <1 kb of upstream sequences are sufficient to confer correct tissue-specific expression and induction by environmental stimuli. Here we describe a translational fusion of a 494-bp promoter fragment of the GRP 1.8 gene and the β-glucuronidase (GUS) reporter gene (Jefferson, 1987; Jefferson *et al.*, 1987). We found that this small GRP 1.8 promoter fragment gives a very specific developmental pattern of GUS expression in vascular tissue of transgenic tobacco plants. This pattern of gene expression largely coincides with the cell-type-specific pattern of accumulation of GRP 1.8 protein in bean as previously determined by immunolocalization studies (Keller *et al.*, 1989). The gene fusion is also excision-wound induced in transgenic tobacco in a specific set of cells close to the wound site. This induction is more rapid than in bean, indicating a difference in wound signal transduction in the two plants.

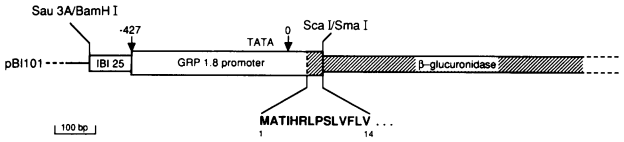

Fig. 1. Structure of the GRP 1.8 promoter–GUS fusion in the plasmid vector pBI101.1. The construct contains 427 bp of sequences upstream of the transcription start site and results in a translational fusion. The first 14 amino acids of the GUS fusion protein are identical with the GRP 1.8 sequence. The 5′ untranslated leader sequence which contains several repetitive sequences (Keller *et al.*, 1988) is therefore completely included in the construct.

Table I. Expression of GUS under control of the GRP 1.8 promoter in transgenic plants

	Plant						
	1	2	3	4	5	6	Control[b]
Leaves[a]	660	374	836	451	2160	4200	8
Stems	22	44	200	117	408	1080	11
Roots	2080	660	3850	1650	17	5640	33

Fluorescence was measured as described by Jefferson *et al.* (1987) and GUS activity expressed as pmol of 4-methyl umbelliferone/min/mg of protein.
[a]All plants examined were ~8 cm high and grown in sterile containers.
[b]A plant transformed with the promoterless plasmid pBI101.1.

Results

Transformation of tobacco with a GRP 1.8–β-glucuronidase translational fusion

A 494 bp fragment of the GRP 1.8 promoter was fused to the β-glucuronidase (GUS) reporter gene (Jefferson *et al.*, 1987). This fragment contains a sequence of 427 bp upstream of the transcriptional start site (Figure 1). The construct results in a translational fusion, with the fusion protein containing the first 14 amino acids of the GRP 1.8 protein followed by the GUS sequence (Figure 1). This construct was transformed into tobacco using leaf disc transformation. Plants were regenerated and then analyzed for GUS expression. Six plants showing GUS expression were selected for further analysis.

Developmental and tissue-specific regulation of the GRP 1.8 promoter

Leaves, roots and stems of transgenic plants were analyzed for GUS activity (Table I). Within individual plants, roots had a 1.3- to 5-fold higher GUS activity than young leaves, and a 5- to 90-fold higher activity than stems. Between the different transformants, expression in the same organ differed up to 50-, 12- and 9-fold in stems, leaves and roots respectively. While position effects certainly play a role in these variations, part of these differences might also be the result of slightly different ages and developmental stages of

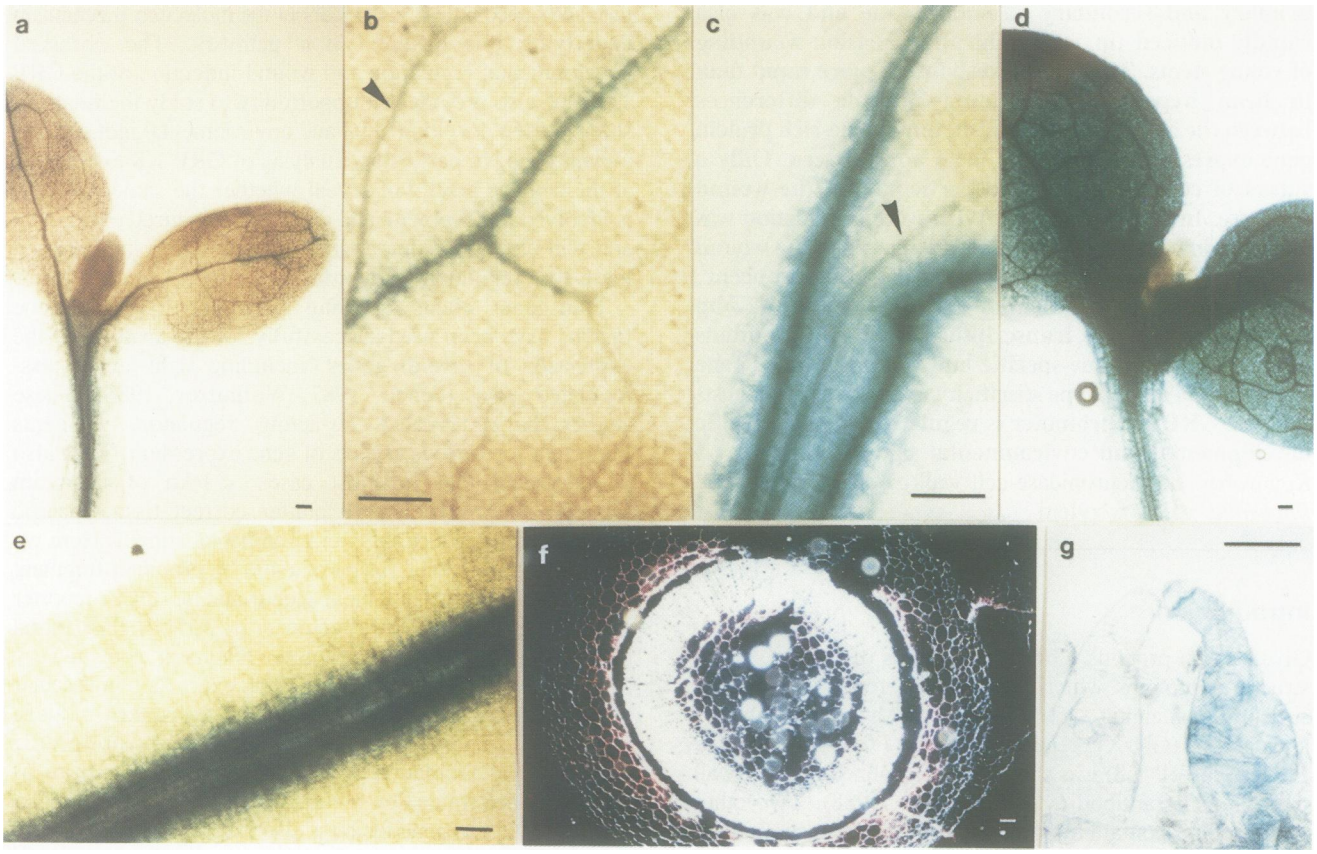

Fig. 2. Histochemical localization of GUS activity during development of transgenic plants containing the GRP 1.8 promoter. (**a**) Cotyledons and upper part of the hypocotyl of a 6-day-old seedling. (**b**), (**c**) Higher magnifications of cotyledons and hypocotyls. In (**d**) the cotyledons of a control plant containing the CaMV 35S promoter fused to the GUS gene are shown. This positive control plant was transformed with the plasmid pBI221 (Jefferson *et al.*, 1987). (**e**) Longitudinal section of a root. (**f**) Transverse stem section of a plant ~5 cm high (viewed in darkfield). (**g**) Cell culture established from a transgenic plant. The bars represent 100 μm.

the plants. In one transgenic plant, there was no expression in roots at all.

To study the expression of GRP 1.8 during development, seeds of selfed transgenic plants were germinated and then histochemically stained for GUS activity. Figure 2a shows an overview of the cotyledons and the upper part of the hypocotyl of an etiolated seedling, the blue color indicating GUS activity. In Figure 2b and c parts of the cotyledons and the hypocotyl are shown at higher magnification. Thus, these plants strongly express GRP 1.8 in primary vascular tissue. Very young vascular tissue (marked by arrows in Figure 2b and c), where a single future tracheary element is distinguishable, already expresses the gene. In cotyledons, the staining is often stronger close to the main vein. However, this may merely reflect a longer period of accumulation of GUS activity in these older cells. Some young, differentiating tracheary elements distant from the main vein in cotyledons do not yet accumulate visible stain (Figure 2b). This staining pattern in cotyledons suggests that a vessel element is morphologically recognizable as such before strong GRP expression starts. GRP 1.8 promoter expression is also seen in the vascular strand leading to very small leaves which have just started to differentiate (Figure 2b).

There was no such confined histochemical reaction in hypocotyls of control plants expressing the GUS gene under control of the CaMV 35S promoter (Figure 2d). In these plantlets, GUS was uniformly expressed in the cotyledons and also in the hypocotyl.

In roots and stems of axenically grown plants, GRP 1.8 promoter expression is also confined to the vascular tissue (Figure 2e and f). In tobacco, stems of small plants have already undergone secondary thickening. Sections of histochemically stained and paraffin embedded stems were observed in darkfield in a microscope. In darkfield, the histochemical stain in thin sections was more clearly visible than in brightfield. Under these conditions, the GUS histochemical staining appears light red. In stems, a ring of cells immediately adjacent to the outside of xylem tissue strongly expressed the GRP 1.8 promoter (Figure 2f) and this corresponds to tissue differentiating into secondary vascular elements.

In roots (Figure 2e), expression in vascular tissue was strongest in a region in the lower part, where differentiation of primary vascular tissue occurs (see also Figure 4 below). Furthermore, expression of the GRP 1.8 promoter was observed in the vascular tissue of all other organs examined, including petals, anthers and pistils in the flower.

Expression of the GRP 1.8 promoter in transgenic cell cultures

Differentiation of vascular tissue is controlled or strongly influenced by plant hormones, specifically by auxins and cytokinins (Aloni, 1987). To investigate possible regulation of GRP 1.8 by hormones, we established cell cultures from two transgenic plants carrying the GRP 1.8–GUS construct. These cultures strongly expressed the GRP 1.8 gene (Table II) and high levels of GUS activity were found throughout the batch cell culture cycle following transfer to fresh media. The cell cultures expressing GRP 1.8–GUS gene fusions were grown in a medium containing only the auxin 2,4-dichlorophenoxyacetic acid (2,4-D) as a hormone. No consistent changes were noted when the cytokinin 6-benzyladenine was included in the medium (Table II). Incubation of these cultures with X-Glu for histochemical staining always showed a population of cells staining blue and thus expressing the gene strongly, whereas other cells were not stained (Figure 2g). On a protein basis, the gene was ~4 times more strongly expressed in cell culture than in root tissue. Interestingly, the differences observed in the expression levels of the gene between transgenic plants were maintained in cell culture. These results demonstrate that GRP 1.8 has a different expression pattern in cell cultures from enzymes involved in lignin biosynthesis (Kuboi and Yamada, 1978).

Table II. Expression of GUS under control of the GRP 1.8 promoter in cell cultures derived from transgenic plants

Plant	0.22 mg/l 2,4-D				0.22 mg/l 2,4-D and 0.23 mg/l benzyladenine
	0 days[a]	1 day	4 days	7 days	4 days
3	14 080	10 120	17 600	15 840	6864
4	4620	5456	4400	ND[b]	4488
Control[c]	20	ND	18	ND	ND

GUS activity is expressed as pmol 4-methyl umbelliferone/min/mg of protein.
[a]Days after transfer into fresh medium.
[b]Not determined.
[c]A cell culture derived from a plant transformed with the promoterless plasmid pBI101.1.

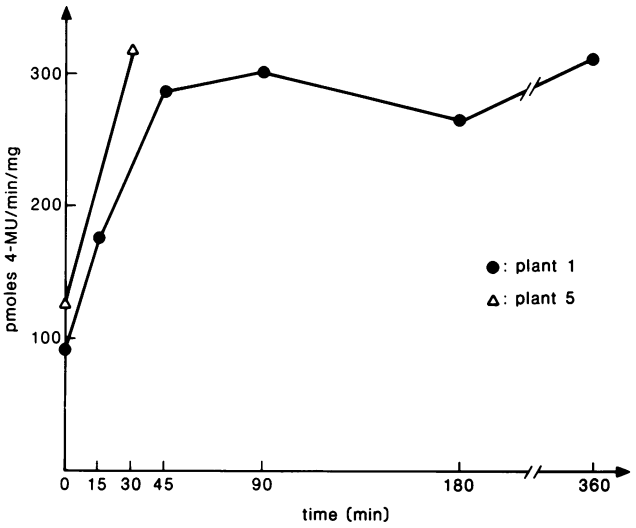

Fig. 3. Wound-induction kinetics of the GRP 1.8 promoter in two different transgenic plants. Stems from plants ~8 cm tall were excision wounded and incubated in buffer for different times prior to extraction and fluorometric assay of GUS activity.

Wound-induction of the GRP 1.8 promoter

The GRP 1.8 transcript has been shown to be wound-induced (Keller *et al.*, 1988). Although wound-induced genes are quite widespread in plants, little is known about cell-type specificity of wound responses. Wound induction was analyzed in transgenic plants containing the GRP 1.8–GUS gene fusion. Wound-excised stems were incubated in buffer and then analyzed for GUS activity. We found about a 3-fold induction after wounding (Figure 3). The magnitude of induction varied between the different plants, probably

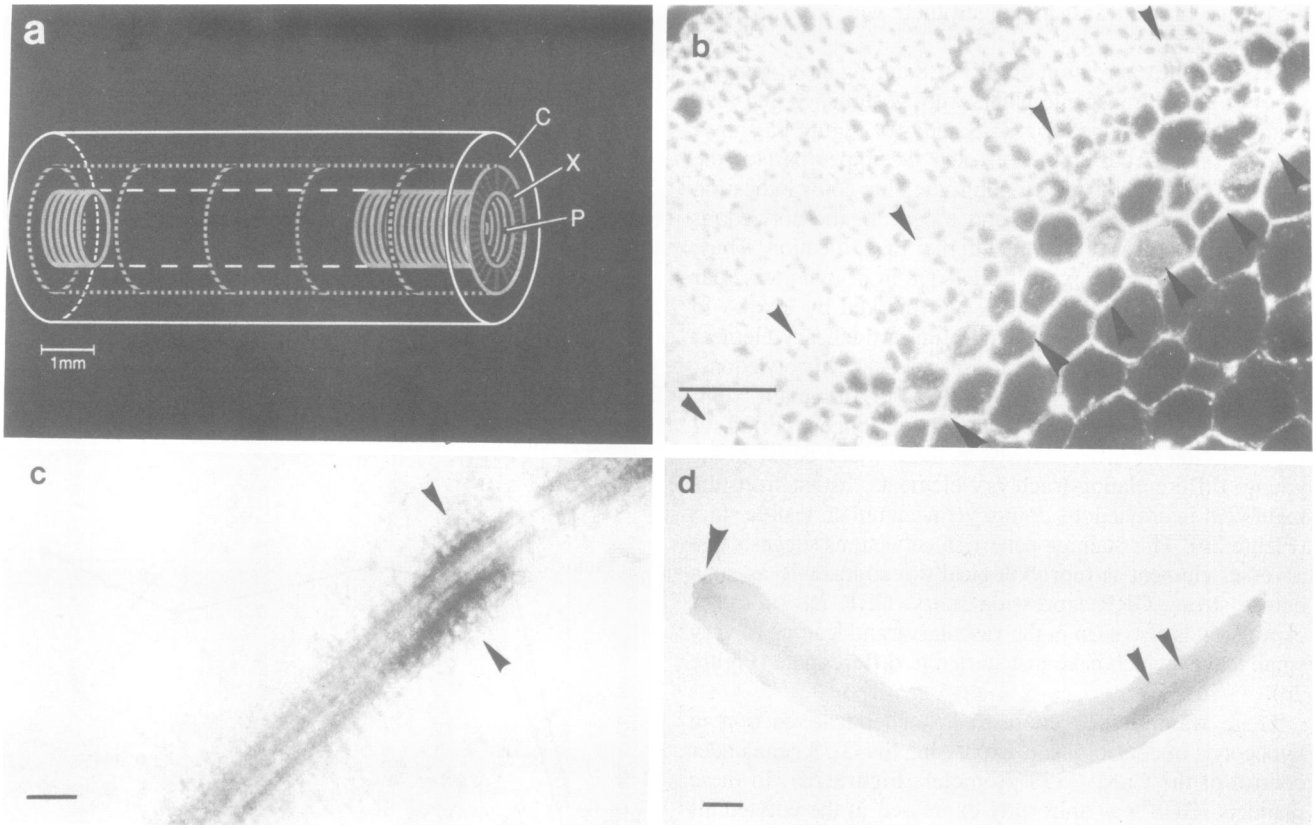

Fig. 4. Histochemical localization of GRP 1.8 promoter induction in wounded tissue. (**a**) Schematic representation of GUS activity in a stem section: p, pith; x, xylem; c, cortex. The dotted cylinder outside the ring of xylem tissue indicates developmental expression of the GRP 1.8 promoter in secondary xylem tissue, the rings inside the xylem show where wound-induced expression is observed. (**b**) Part of a stem section at high magnification, observed in darkfield. In the cells in the area between the two sets of arrows wound-induced expression was observed. (**c**) Wound site where a root was excised from the plant. The arrows mark the sites of GUS expression. (**d**) Root at lower magnification. The arrows point to the wound site and the part of the root where GUS is developmentally expressed. The bars represent 100 μm except in (d), where the bar represents 1 mm.

depending on the level of developmental expression in the secondary xylem (Figure 2e). The induction of GUS activity was very rapid, peaking 30−45 min after wounding. This is a considerably more rapid response than observed for the endogenous GRP gene in bean hypocotyls wounded in a similar manner, where maximal levels of the transcript were not observed until 8 h after wounding (Keller *et al.*, 1988).

Only a small set of cells was found to participate in the GRP wound response. Thus, in pieces of transgenic tobacco stem, wound induction was seen in a ring of cells inside the vascular cylinder in pith parenchyma cells. The drawing in Figure 4a shows this induction pattern in a schematic way. Developmental expression on the outside of the vascular cylinder, probably corresponding to secondary xylem formation, is observed all along the stem; wound-induction is restricted to the pith tissue within 1−2 mm of the wound surface (indicated by rings inside the xylem cylinder). A high magnification of a thin section of a paraffin-embedded wounded stem is shown in Figure 4b. Wound regeneration of vessels is known to start from pith parenchyma cells (Cassab and Varner, 1988). However, we cannot exclude the possibility that the observed expression might also occur in cells of the inner phloem.

In excised roots, a wound response is always visible where the root was cut off from the rest of the plant (Figure 4c). No incubation was necessary to obtain this reaction, presumably because the time that elapses during cutting and fixation is sufficient to obtain the wound-induction. As in stems, wound-induction in roots was also confined to the region immediately adjacent to the damaged surface. The observed pattern of expression is not due to limited penetration of the substrate into the tissue, since internal vascular tissue, where GRP 1.8 is developmentally expressed, is clearly stained (Figure 4d).

RNase protection

To verify that the observed GUS activity is due to specific expression of the GRP 1.8 promoter and not caused by some spurious activation due to other sequences (e.g. co-transferred plasmid sequences), we performed an RNase protection experiment using RNA isolated from cell cultures of a transgenic plant, where GUS activity was highest. With RNA from bean ovaries, two bands were protected (Figure 5a). A strong band, ~60 bases long, probably corresponds to the previously mapped start site in bean hypocotyls (Keller *et al.*, 1988). There is a much weaker protected fragment ~5−8 bases longer. With RNA from cell cultures of a transgenic plant, only one fragment of 70 bases was protected (Figure 5b). By chance the last 3 bases of the pSP64 plasmid vector sequence before the insert and the first 3 bp of the GUS gene are identical, thus giving a protected fragment which is 3 bases longer than the protected fragment from the same start site in bean. The observed protected fragments were not found with RNA from cell cultures of non-trans-

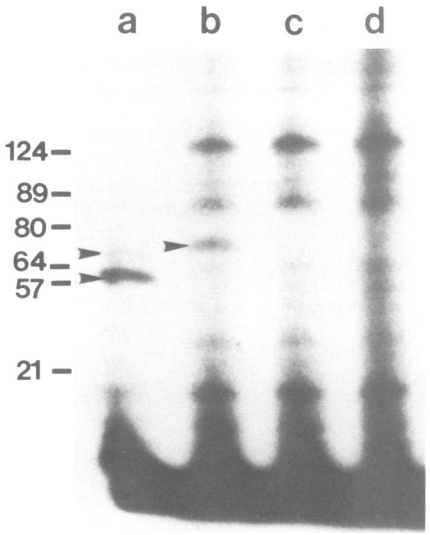

Fig. 5. RNase protection of a fragment spanning the transcription start site of GRP 1.8.Thirty micrograms of total RNA was used for the protection experiments. (**a**) RNA from bean ovaries. (**b**) RNA from a cell culture established from transgenic plant 3. In (**c**) RNA from transgenic tobacco cell cultures transformed with the control plasmid pBI101.1 was used. In (**d**) only tRNA was added. The arrows indicate the protected RNA fragments. The size markers were *Hae*III fragments of pBR322 DNA.

formed plants or controls without any test RNA (Figure 5c and d). Some background bands were visible, the origin of which is unknown. This experiment demonstrates that the transcription initiation in transgenic plants is specific and due to the GRP 1.8 promoter.

Discussion

Tissue-specific and wound-induced expression of the GRP 1.8 promoter: a cell differentiation event

The results presented here demonstrate that the GRP 1.8 promoter is expressed in vascular differentiation during development. Expression was observed in vascular tissue of every organ examined. Thus synthesis and deposition of GRP cell wall protein seems to be a crucial and necessary event during differentiation of a future tracheary element. As we have shown earlier (Keller *et al.*, 1989), GRP 1.8 is closely associated with the secondary wall thickenings in tracheary elements. The observed early expression of GRP 1.8 during differentiation suggests that GRP is laid down early during cell wall thickening, possibly as a scaffold and template for lignin deposition and polymerization, and not merely added at a late stage to a preformed structure.

The GRP 1.8 promoter is also specifically expressed in response to mechanical wounding. We do not yet know if this wound induction reflects differentiation of pith parenchyma cells into wound vessels or if it is part of a general wound reaction. Wounding also induces lignin deposition. Hence, GRPs and lignin might be integrated into the wall of wound response cells in a manner similar to that inferred from the co-localization of GRP and lignin in tracheary elements (Keller *et al.*, 1989). However, in stems of transgenic plants carrying a promoter for phenylalanine ammonia-lyase (PAL), an enzyme involved in lignin biosynthesis (Jones, 1984), wound induction was not seen inside the vascular cylinder but only in certain other tissues such as the epidermis (X.Liang, personal communication). This suggests that the GRP 1.8 wound induction is a differentiation event and not part of a protection response involving lignin deposition.

Wound induction of the GRP 1.8 promoter in transgenic plants was found to be much more rapid than in bean. This probably reflects differences in wound signal transduction and GRP activation mechanisms in the two plants. Thus, the host appears to determine the kinetics of this wound response. Another example where the kinetics of gene induction are determined by the host is the induction of the soybean conglycinin gene in soybean and transgenic petunia (Beachy *et al.*, 1985). Alternatively, additional *cis*-acting regulatory sequences that modulate the kinetics of the wound-induced transcription of GRP may be located outside of the promoter fragment used in the present gene fusion. However, it has been shown in petunia that endogenous GRP wound induction is very rapid compared to bean (Condit and Meagher, 1987), thus establishing that different plants have different kinetics for induction of this gene.

Wounding induces the transcription of a battery of plant genes encoding proteinase inhibitors (Graham *et al.*, 1985), PAL and other enzymes of phenylpropanoid biosynthesis (Edwards *et al.*, 1985), thionins (Bohlmann *et al.*, 1988), HRGPs (Chen and Varner, 1985) and a number of other polypeptides in addition to GRP. However, it is clear from the differences in kinetics and spatial patterns of activation of individual genes that a complex interplay between cell-type-specific developmental cues and an array of wound signals is involved in wound induction. Hence, wound activation of the GRP 1.8 promoter in pith tissue immediately adjacent to the wound surface may reflect rapid accumulation of hormones or other wound signals at this site triggering induction in cell types developmentally competent for GRP 1.8 expression.

Expression of the GRP 1.8 promoter in cell culture

The observed expression of the GRP 1.8 promoter in cell cultures containing only the auxin 2,4-D as a hormone suggests a simple hormonal requirement for expression of this gene. A cell culture system for the differentiation of tracheary elements has been described for tobacco (Kuboi and Yamada, 1978). In that system, tracheary elements were formed only after addition of a cytokinin to a culture growing with auxin. However, GRP 1.8 expression does not require cytokinins in culture and this is not dependent on formation of tracheary elements in tobacco. In bean it has been shown that with auxin alone, xylem tissue can be induced in callus culture (reviewed by Lamb, 1981). The strong expression in cell culture observed here, which is at least 4 times higher than in any other plant tissue, provides a useful system for isolation and characterization of nuclear protein factors interacting with the promoter.

The GRP 1.8 promoter is a model for gene expression in vascular tissue

A short 427 bp fragment upstream of the GRP 1.8 transcription start site is sufficient for tissue-specific gene expression in vascular tissue and for wound-induction. In this case, no 3′ sequences seem to be necessary for wound-induction, in contrast to the potato proteinase inhibitor II gene, which is also wound-induced (Thornburg *et al.*, 1987). In future studies the GRP 1.8 promoter will be

analyzed in detail for elements involved in developmental regulation and wound induction. This would ultimately lead to an understanding of the molecular events underlying the differentiation of tracheary elements.

Materials and methods

Plasmids, gene constructs and plant transformation

A plasmid obtained during nucleotide sequence analysis (Keller *et al.*, 1988) contains 427 bp upstream of the transcription start site of GRP 1.8. A *Sau*3A–*Sca*I fragment (the *Sau*3A site is in the bacterial plasmid pIBI25) was cloned into pIB101.1 (Jefferson *et al.*, 1987). The translational fusion was sequenced using the dideoxy sequencing method (Sanger *et al.*, 1977) with a β-glucuronidase sequence specific primer (Clontech, Palo Alto, CA). This construct (pGRP180) was mobilized from *Escherichia coli* HB101 into *Agrobacterium tumefaciens* LBA 4404 as described by Bevan (1984). Leaf discs of *Nicotiana tabacum*, var. Xanthi were transformed as described by Horsch *et al.* (1984). Transformed plants were selected on Murashige and Skoog medium (Murashige and Skoog, 1962) containing 200 μg/ml kanamycin and 500 μg/ml carbenicillin or cefatoxim.

RNase protection experiments

Total cellular RNA was isolated as previously described (Bell *et al.*, 1986). The plasmid used to generate a labeled probe was pSP64 containing the same fragment of bean GRP 1.8 promoter DNA which was used for the promoter fusion to the GUS reporter gene. *In vitro* transcription was performed as described by Melton *et al.* (1984). RNase protection experiments were performed using 30 μg of total RNA according to Zinn *et al.* (1983).

β-Glucuronidase assay and histochemical localization

Tissue extracts were prepared and analyzed for fluorescence of the reaction product 4-methyl umbelliferone as described by Jefferson *et al.*, 1987, using 4-methyl umbelliferyl glucuronide (Sigma) as a substrate. Usually, the reaction was performed for 60 min at 37°C and stopped with 4 vols of 0.2 M Na_2CO_3. Histochemical assays were also performed as described by Jefferson *et al.* (1987). After a light fixation with formaldehyde (0.3%), the tissue was incubated with X-Glu (Research Organics Inc., Cleveland, OH). After histochemical staining and post-fixation with 2% glutaraldehyde for 60 min, stems were embedded in paraffin as described by O'Brien and McCully (1981). Paraffin sections were then observed in the darkfield mode with a Nikon Diaphot TMD microscope and micrographs were taken on Kodak Tungsten 50 film.

Excision wounding of stems

Stems from axenically grown transgenic plants were cut into 3–5 mm long pieces and then incubated in sterile 5 mM phosphate buffer (pH 7.0).

Establishment of transgenic cell cultures

Leaves from two transgenic plants were cut into small pieces and then incubated for 18 days in liquid LS medium (Dixon, 1985), containing 0.22 mg/l 2,4-D. The resulting culture was subcultured five times before analysis.

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