

Bean ribonuclease-like pathogenesis-related protein genes (*Ypr10*) display complex patterns of developmental, dark-induced and exogenous-stimulus-dependent expression

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The intracellular pathogenesis-related (PR) proteins of common bean (*Phaseolus vulgaris* L.) are encoded by a highly polymorphic family of at least 20 genes. One member, the *Ypr10*c* gene, has been isolated and characterised. The deduced amino acid sequence of the encoded protein, PR-10, exhibits similarities to tree-pollen allergens, to food allergens from celery and apple and to ginseng ribonuclease peptide sequences. We show by RNA blot analysis that the *Ypr10* gene family, including *Ypr10*c*, is strongly expressed in bean roots. In leaves *Ypr10* transcript levels are low in young and mature stages but are elevated during senescence and in diseased states. Dark treatment of leaves causes strong induction of *Ypr10* transcripts, which is reversible by light, and diurnal rhythms of transcript accumulation during the night are observed. *Ypr10* genes are responsive to external stimuli related to pathogen-defence such as glutathione or salicylic acid. Transcriptional activity of a *Ypr10*c* promoter– β -glucuronidase fusion gene in transgenic tobacco was observed in roots, in developing xylem and phloem of stems, and in the blade of senescent leaves, with highest levels at the onset of senescence. The most striking characteristic of developmental expression was the specific localisation of β -glucuronidase activity in the transmitting tract of styles in flowers at anthesis. Feeding of various pathogen-related and stress-related stimuli to young tobacco leaves led to accumulation of GUS activity in leaf blades. We identify considerable spatio-temporal similarities between reported expression patterns of *Ypr10* genes and ribonuclease genes, which, together with the significant sequence similarity to the ginseng ribonuclease, support the hypothesis of a ribonuclease function for PR-10 proteins and allow the prediction of possible biological roles.

Keywords: allergen; allergy; *Phaseolus vulgaris* L.; ribonuclease.

Pathogenesis-related (PR) proteins are plant proteins induced in pathological or related situations. This induction implies a role in pathogen restriction and disease resistance, yet the biochemical and physiological nature of their contribution to pathogen defence is largely unknown (reviewed by Cutt and Klessig, 1992; Stintzi et al., 1993). The term PR protein has recently been extended to include all proteins, of known or unknown function, that are involved in the response of a plant to a pathogen. Consequently, a further six families have been added to the five well-established families of PR proteins (Van Loon et al., 1994). One of the characteristics of members of the classical five PR protein families is their accumulation in the extracellular space or the vacuole. Furthermore, their gene organisation and their differential expression is highly complex (Cutt and Klessig, 1992; Stintzi et al., 1993).

Intracellular PR proteins were first described from parsley cell suspension cultures treated with fungal elicitor (Somssich et

al., 1986). They have subsequently been shown to be ubiquitous in the plant kingdom (Walter et al., 1990). Intracellular PR proteins are structurally related to tree-pollen allergens and to major food allergens from celery and apple (Breiteneder et al., 1989, 1995; Vanek-Krebitz et al., 1995), but are unrelated to any of the classical PR proteins, despite their initial description as PR-1 proteins (Somssich et al., 1986; Walter et al., 1990; Warner et al., 1992). Following the recently established nomenclature, the intracellular PR proteins are now called PR-10 (genes are termed *Ypr10*), and PvPR1 and PvPR2 from *Phaseolus vulgaris* L. are now termed PR-10a and PR-10b, respectively (Van Loon et al., 1994; Walter et al., 1990). The precise cellular localisation of PR-10 proteins has not been determined but the absence of apparent signal peptides and the identity of cDNA-derived amino acid sequences with those obtained from protein sequencing indicate that PR-10 proteins are cytosolic (Awade et al., 1991; Walter et al., 1990). Their size is remarkably invariable (154–160 amino acids, 16–18 kDa). Several research groups have reported local accumulation of PR-10 mRNAs or *Ypr10*–promoter activity at sites of pathogen encounters or wounding (Schmelzer et al., 1989; Somssich et al., 1988; Warner et al., 1992, 1993). However, overexpression of a *Ypr10* gene caused no improvement in disease resistance (Constabel et al., 1993).

There is growing evidence that many, if not all, PR proteins have important, albeit often unknown, functions in plant development. Roots and senescent leaves have been shown to be sites of developmental accumulation of classical PR proteins (Castre-

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Abbreviations. *Api* g 1, major allergen from celery; *Bet* v 1, major allergen from birch pollen; PR, pathogenesis related; GSH, glutathione; X-Glu, 5-bromo-4-chloro-3-indolyl- β -D-glucuronide.

Note. The novel nucleotide sequence data published here have been submitted to the EMBL sequence data bank and are available under accession number X96999.

sana et al., 1990; Grüner and Pfitzner, 1994). Appearance during normal flower development is another feature found repeatedly for PR-1, PR-2 and PR-3 proteins (Lotan et al., 1989; Ori et al., 1990). There are reports on significant endogenous levels of PR-10 proteins, their mRNAs or promoter activity in seeds (Barratt and Clark, 1991; Warner et al., 1994) roots, senescent leaves (Crowell et al., 1992), stems (Warner et al., 1994) and various parts of flowers (Breiteneder et al., 1989; Constabel and Brisson, 1995; Swoboda et al., 1994; Warner et al., 1993, 1994).

Biochemical functions of PR proteins are being elucidated. PR-2 proteins and PR-3 proteins have been identified as β -1,3-glucanases and chitinases, respectively (Kauffmann et al., 1987; Legrand et al., 1987). Intracellular PR-10 proteins might be ribonucleases based on the significant amino-acid-sequence similarity of two ginseng ribonuclease peptides to parsley PR-10 proteins (Moiseyev et al., 1994). This finding led to a preliminary classification of PR-10 proteins as ribonuclease-like PR proteins (Van Loon et al., 1994). Here we report the isolation, structural characterisation and genomic organisation of one member of the *P. vulgaris* L. *Ypr10* gene family. We used gene-specific and non-specific probes on bean RNA samples and a promoter- β -glucuronidase gene fusion in transgenic tobacco to perform a fairly comprehensive survey of expression patterns of bean *Ypr10* genes. We corroborated a number of regulatory features previously observed in other systems and identified additional distinct patterns of developmental and inducible expression. Taken together, the regulatory data now available for *Ypr10* genes exhibit a remarkable degree of similarity with known regulatory features of ribonucleases.

MATERIALS AND METHODS

Plant material and treatments. *P. vulgaris* L. (cv. Procesor) plants were grown to maturity (4–5 weeks) in a greenhouse. Leaf or stem material was harvested into liquid nitrogen. Roots were washed extensively with tap water and deionized water, then briefly dehumidified in an air stream prior to harvest. Approximately 170 flowers at, or shortly after, anthesis were dissected for preparation of sepals (400 mg), pistils (500 mg) and stamens (50 mg). Deprivation of light during dark treatments was accomplished by covering plants with large pots, which contained little openings for air circulation. Therefore, a very small amount of residual light was present. Nocturnal measurements of transcript levels were carried out in June in a greenhouse with very low background light levels during the night. Feeding of exogenous stimuli was performed by immersion of leaf stalks connected to intact, young, fully expanded leaves into 20 mM sodium phosphate, pH 5.8, which contained the stimuli. Salicylic acid was used as sodium salt (Sigma). Local application of stimuli was carried out by addition of inducing solutions or controls to wound sites created by removal of discs from detached leaves with a pipet tip. Treated leaves were incubated in a moist atmosphere at 25°C for 40 h prior to further analysis.

Gene isolation and structural characterisation. The construction and the screening procedure of the *P. vulgaris* L. λ EMBL3 genomic library has been described (Walter et al., 1994a). ^{32} P-labelled EIT23 (PvPR1) insert DNA (Walter et al., 1990) was used as a probe in screening. Suitable fragments for DNA sequencing, as determined by a mapping analysis of λ DNA, were subcloned into the pBS (+/–) plasmid vector (Stratagene). dsDNA sequencing by the dideoxynucleotide chain-termination method was performed on the plasmid subclones by T7 DNA polymerase (Pharmacia). A nested-deletion strategy (Pharmacia) was employed to obtain internal-sequence

data. Second-strand-sequence data were collected by means of synthetic oligonucleotide primers. The Heidelberg Unix Sequence Analysis Resources (HUSAR) programme package was used for sequence analysis. Alignments were created by means of the GAP routine and occasionally further optimised manually.

A 20-base oligonucleotide, 5'-CTTGAAGGAATCAACGG-CCT-3'; and poly(A)-rich RNA from glutathione-induced bean leaves were combined in a primer-extension reaction to determine the transcription-start site. Priming and extension were carried out by a first-strand-synthesis kit (Amersham) supplemented with [^{35} S]dATP[S] for radioactive labelling of the newly synthesised strand. The sizes of resultant cDNA fragments were determined on a sequencing gel next to a DNA-sequencing reaction primed by the same oligonucleotide.

DNA extraction, RNA extraction, blotting and hybridisation. The isolation procedures for bean genomic DNA and bean RNA and methods for blotting to Genescreen Plus membrane (Dupont) have been described (Walter et al., 1994a; Schaaf et al., 1995). Equal loading of RNA on gels was routinely checked by means of ethidium-bromide-stained gels. Hybridisations at high stringency were carried out as previously reported (Walter et al., 1994a; Schaaf et al., 1995) or in Quickhyb hybridisation solution (Stratagene) at 68°C for 2 h according to the manufacturers instructions. Final washes were in 15 mM NaCl, 1.5 mM sodium citrate, pH 8.0, at 65°C or at 60°C, respectively, for 30 min. Moderate-stringency washes were at 50°C in the same buffer for 30 min.

β -glucuronidase gene fusion construction and tobacco transformation. The *Ypr10**c promoter was excised from a 3.1-kb *EcoRI*-cloned genomic fragment by *EcoRI* and *EarI* (Fig. 1a). The *EarI* site is located 41 bp upstream of the translation-start site, which results in the representation of part of the leader in the final construct. The 2480-bp *EcoRI*/*EarI* fragment was transcriptionally fused to the β -glucuronidase reporter in the binary vector pBI101.4 cleavage of the vector with *XbaI* and *BamHI*, filling in of the *BamHI*-cleaved end dGTP such that the end is complementary to the *EarI*-cleaved end in the promoter fragment. This strategy ensured correct fusion of the promoter to the β -glucuronidase gene. After filling in of the residual ends, the plasmid was circularised by blunt-end ligation. The fusion was verified by restriction analysis and by DNA sequencing of fusion borders using a β -glucuronidase-gene-specific primer (Clontech). Conditions for transfer to *Agrobacterium tumefaciens* LBA 4404, transformation of *Nicotiana tabacum* cv. Petit Havana SR1, and maintenance of transformants have been described (Schaaf et al., 1995). DNA blot analysis of individual transformants demonstrated the incorporation of several gene copies into the tobacco genome in each experiment (data not shown). Transformants that represent high (PR-GUS1), intermediate (PR-GUS12, PR-GUS13) and low (PR-GUS4) expression levels, based on β -glucuronidase assays of roots, were chosen for further analysis. Transgenic tobacco plants created previously in our laboratory (Schaaf et al., 1995), which contained either a promoterless β -glucuronidase gene or the β -glucuronidase gene fused to the cauliflower-mosaic-virus 35S promoter were used as controls. Patterns of *Ypr10**c promoter activity were analysed in the T_1 generation. Some variation in expression levels, but not between different T_1 plants from a primary transformant, was observed. No evidence for or against transgenic silencing was obtained. The PR-GUS12-2B T_1 progeny plant, which showed a high expression level compared with some of the other PR-GUS12 progeny plants, was chosen for clonal propagation and detailed quantitative analysis in selected experiments.

β -glucuronidase assays. β -glucuronidase activity in crude protein extracts was determined fluorometrically as described

(Jefferson, 1987). Fluorescence of the 4-methyl-umbelliferone produced by β -glucuronidase activity was measured on a DNA fluorometer TKO 100 (Höfer). Data are presented as the mean of β -glucuronidase activities (and SD where appropriate) from at least two independent determinations. The values were corrected for a low intrinsic degradation of the substrate (4-methyl-umbelliferyl-glucoside), which was 20–60 pmol 4-methyl-umbelliferone $\cdot$ min $^{-1}$ $\cdot$ mg $^{-1}$ protein for 60-min incubation. This background level was seen initially with SR1 wild-type tobacco or promoterless controls but was subsequently also observed when protein extract was completely omitted, which indicates that there is virtually no detectable endogenous activity in vegetative tissues of SR1 tobacco plants. Protein content of sample extracts was determined by the Bio-Rad Protein Assay Dye Reagent with BSA as a standard.

Histochemical staining for β -glucuronidase activity was performed essentially as described (Jefferson, 1987) with X-Glu (5-bromo-4-chloro-3-indolyl- β -D-glucuronide) (Biosynth AG or Clontech) as a substrate. Flower tissues were dissected before further manipulations. Pistils, ovaries and seed pods were bisected prior to staining to allow penetration of the substrate. Staining of pollen was performed while still bound to anthers or after removal from anthers. No difference between these two staining methods was observed. Hand sections or intact organs were vacuum infiltrated for a few minutes with 1 mM X-Glu in 50 mM sodium phosphate, pH 7.0, 0.02% Triton X-100 and 0.2–0.5 mM each of $K_3[Fe(CN)_6]$ and $K_4[Fe(CN)_6]$ and incubated at 37°C for 3–15 h. Stained tissue was cleared of chlorophyll in 70% ethanol prior to visual analysis using a stereomicroscope SV11 (Zeiss). Documentation was in bright-field or dark-field microscopy on colour slides.

RESULTS

Isolation and structural characterisation of the *Ypr10*c* gene.

A bean genomic library was screened with the EIT23 cDNA probe (Walter et al., 1990). A positive clone (gPvPR1) that contained a 14-kb *SalI* insert was purified, and characterised by restriction mapping. The *Ypr10* gene and flanking regions were located in a region of approximately 6.4 kb (Fig. 1a). Several overlapping restriction fragments from this region were subcloned and fully or partly sequenced. The promoter and leader sequences determined are shown to the ATG translation-start codon in Fig. 1b. Determination of the transcription-start site gave one major and two minor signals (Fig. 1b). We consider it likely that the two minor signals represent binding and extension of the primer to transcripts of highly similar *Ypr10* genes rather than minor start sites of transcription. A TATA box (TA-TAAATA) was identified at position –33. An AC-rich H-box-like element, which has been implicated in the regulation of plant defence genes and of *Ypr10* genes, is located at position –51 (Fig. 1b; Warner et al., 1994; Yu et al., 1993). The cloned gene does not correspond to either of the two transcripts characterised previously, but it is 94% identical in the coding region to the cDNA that encodes PR-10b (Walter et al., 1990). The deduced protein is therefore designated PR-10c (96% identical to PR-10b) and the gene is called *Ypr10*c* accordingly. The coding region is interrupted by a single intron of 103 bp at a position conserved in other *Ypr10* genes (Chiang and Hadwiger, 1990; Matton et al., 1990; Meier et al., 1991; Warner et al., 1993).

*Ypr10*c* is a member of a highly polymorphic family of bean genes. The *Ypr10* gene family has previously been estimated to contain at least 12 genes (Walter et al., 1990). Based on the

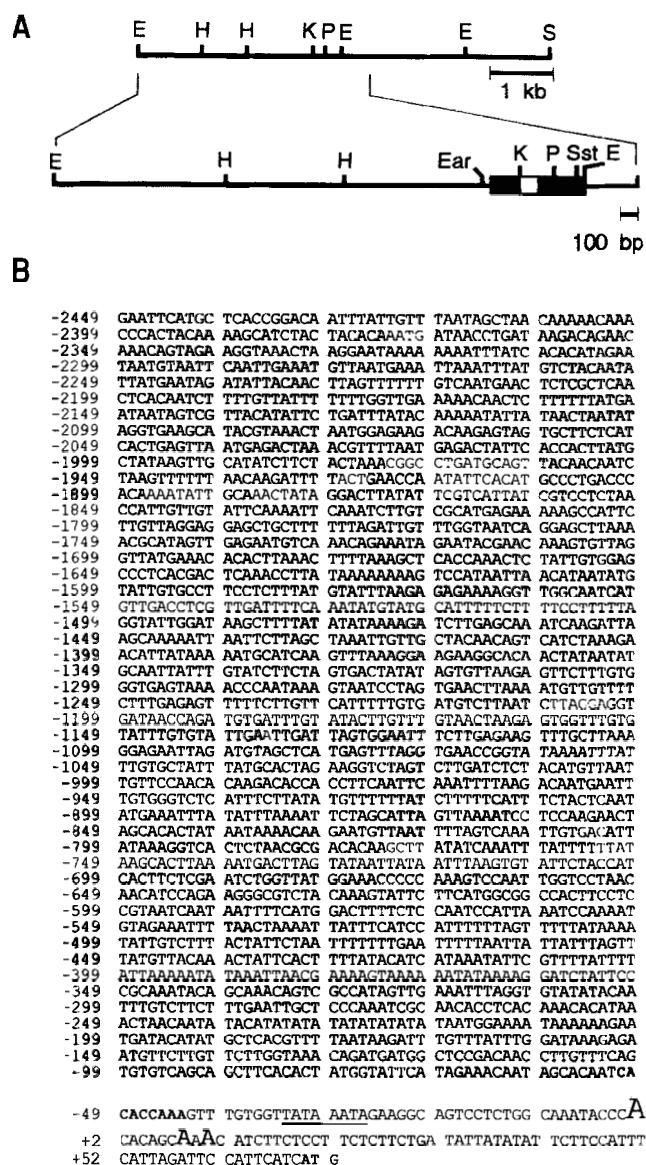


Fig. 1. Structural characterisation of the bean *Ypr10*c* gene. (a) Restriction map of a genomic fragment from bean that contains the *Ypr10*c* gene and flanking sequences (top). The regions that were fully sequenced are enlarged (below). Black boxes represent coding regions in two exons; the open box represents an intron. Restriction enzymes are abbreviated as follows: E, *EcoRI*; Ear, *EarI*; H, *HindIII*; K, *KpnI*; P, *PstI*; S, *SstI*; Sst, *SstI*. (b) Nucleotide sequences of promoter and leader regions of the *Ypr10*c* gene. The major transcription-start site is indicated by a bold, greatly enlarged letter; two putative minor sites are in bold and enlarged. The TATA box is underlined. A possible *cis*-acting element and the ATG translation-start codon are in bold.

genomic sequence of *Ypr10*c* and the restriction-mapping information, we have reinvestigated the size of the *Ypr10* gene family. Particular fragments created by single or combined digestions with restriction enzymes could be assigned to the cloned *Ypr10*c* gene by the size calculated from the DNA sequence (*EcoRI*, 3.1 kb; *PstI/HindIII*, 1.2 kb; *PstI/EcoRI*, 2.9 kb and 0.2 kb; *PstI/HindIII*, 1.4 kb; Fig. 2). The multitude and varying intensity of additional signals on the autoradiograph suggests that at least 20 *Ypr10* genes are present in the bean genome.

PR-10c shares highly significant sequence similarity with a ginseng ribonuclease. High sequence similarity between a ribonuclease from ginseng callus and the parsley PR-10 proteins has

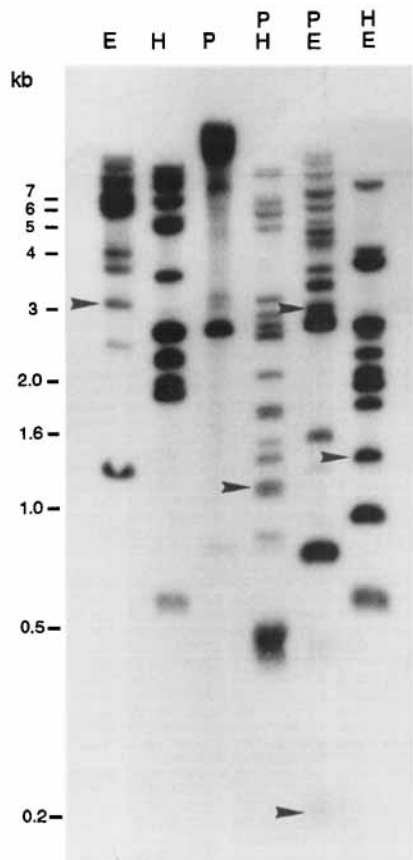


Fig. 2. Genomic DNA blot analysis of *Ypr10* sequences in the bean genome. Bean genomic DNA (10 µg) was digested with single restriction enzymes or combinations of enzymes as indicated. E, *Eco*RI; H, *Hind*III; P, *Pst*I. The blot was hybridised with the EIT14a probe (Walter et al., 1990) under moderate-stringency conditions. Fragments assigned to the *Ypr10*c* gene are marked by arrowheads. The positions of DNA size markers are indicated.

been reported (Moiseyev et al., 1994). We collected the amino acid sequences of known PR-10 proteins, excluding minor variants, and aligned them with the ribonuclease sequence. The PR-10c sequence obtained in the present work and a recently published sequence of the major celery allergen were included. Only 12 residues are strictly conserved in all PR-10 proteins (Fig. 3). The published ginseng ribonuclease peptide sequences do not correspond to any of the strictly conserved regions. However, the N-terminal peptide exhibits 40% identity to PR-10c and only 4 of 20 amino acids are unique to the ginseng sequence, if all PR-10 sequences are compared. Similarly, 45% identity to PR-10c is observed in the internal peptide with only two amino acids unique to the ginseng ribonuclease. In addition, sequence information from peptide sequencing of approximately 70% of the ginseng ribonuclease protein were recently communicated (Moiseyev et al., 1995). At least 8 of the 12 strictly conserved PR-10 residues described above are found in the ginseng protein at the respective positions, which demonstrates the highly significant sequence similarity between PR-10 proteins and the ginseng ribonuclease.

Expression of *Ypr10*c* or other *Ypr10* genes is regulated by developmental cues, by dark treatments and by a diurnal rhythm. The expression patterns of *Ypr10*c* and of structurally similar *Ypr10* genes were analysed by Northern blot analysis of bean RNA samples. The 3' untranslated region of the *Ypr10*c* gene was the only region to exhibit major differences to the EIT

transcripts described (Walter et al., 1990) and therefore appeared to provide a useful gene-specific probe. A 2.0-kb *Eco*RI fragment that contained part of the TGA translational-stop codon and downstream sequences (Fig. 1a) was used as a probe in a bean-genomic-DNA blot-hybridisation experiment. The probe detected a 2.0-kb *Eco*RI fragment as expected and one minor higher-molecular-mass fragment (data not shown), which indicated that the probe could be used specifically for the *Ypr10*c* gene. The EIT14a cDNA, which is 94% identical to the *Ypr10*c* gene in the coding region but excludes 3' untranslated sequences (Walter et al., 1990) was used as a non-specific probe for detection of all *Ypr10* gene transcripts.

RNA from young leaves, mature stems and roots of mature, healthy bean plants grown in a greenhouse was isolated to perform preliminary studies on organ-specific expression. In poly(A)-rich RNA samples, the *Ypr10*c*-specific and the non-specific (*Ypr10*) probe gave a strong hybridisation signal only with root material (Fig. 4a). However, to obtain a signal of similar intensity with the gene-specific probe longer exposure times (fourfold) and a higher-specific-activity probe (threefold) were required, which indicates that the *Ypr10*c* gene is probably not as strongly expressed as other members of the gene family. The basic organ distribution of expression of all *Ypr10* genes appeared to be similar.

In flowers at or shortly after anthesis the *Ypr10* probe detected transcripts in sepals and in the pistil but not in stamens (Fig. 4a). If vegetative tissues of different developmental stages were analysed, the lower and lowest leaves from healthy plants and occasionally occurring malformed or diseased leaves (slightly chlorotic, necrotic or curly, but viable) accumulated *Ypr10* transcripts. A sample of a young stem gave a weak positive signal with the *Ypr10* probe. Strong expression was observed in two independent root samples (Fig. 4b). The ratio of transcript abundance between lower leaves and lowest leaves was quite variable in several duplications of the stage-specific expression analysis, which suggests fine-tuned regulation during maturation and senescence.

In a series of experiments to investigate the effect of light on inducible-expression patterns, we were surprised to find an effect of darkness by itself in leaves. This effect was demonstrated in an experiment under slightly reduced-stringency conditions, which showed strong upregulation of the 800-base transcripts after 48 h of darkness but no effect on a crosshybridising 1500-base leaf-constitutive transcript (Fig. 4c). We analysed the kinetics of dark accumulation of *Ypr10* mRNAs. Darkness imposed during the normal daylight period led to a detectable increase in transcript levels after 12 h of treatment (Fig. 4c). The 24-h dark period did not lead to a greater increase compared with the shorter treatment. However, the 24-h dark treatment involved a period of natural darkness during the night and the onset of the treatment was shifted to a later time during the day, which resulted in a harvest time during midmorning rather than at dusk as in the 12-h time point. A factor related to the time of day when the experiment was started or finished thus apparently affected the regulation. This phenomenon is under further investigation. After 48 h and 72 h of continuous darkness we observed very strong accumulation of *Ypr10* transcripts (Fig. 4c). If plants that were kept in the dark for 48 h were returned to daylight, the *Ypr10* transcripts gradually disappeared within 24 h. Because of the daytime-related variations in dark induction, we analysed a possible diurnal cycle of transcript accumulation during the night. Young and mature bean plants showed elevated *Ypr10* transcript levels during the night, which disappeared at dawn (Fig. 4c). An increase in transcript levels at 22.00, which almost coincided with sunset at the season in which the experiment was performed (June), may indicate that

	30	60 ▼	90
Bean PR-10a	MGVFTFEDQTTSPVAPATLYKAVAKDADTIFPKALPDSFKSVEIVEGNGGPGTIKKISFVEDG-ETKFLVHKIESIDEANLGYSSYSIVGGV		
PR-10b	MA*****LV*****V*****V*****E*****A*****A		
PR-10c	MA*****LV*****N*****V*****L*****G*****A		
SoybeanSAM22	M*****EIN*****N*****N*****T*****L*****V*****A		
H4	M*****E*****LVT*****NVI*****V-EA*****R*****NL*****T*****S*****V*****V*****V*****		
Pea I49	M*****NV*****EI*****V*****I*****LVT*****NLT*****VI*****AI*****I*****A*****LT*****H*****V*****LV*****A*****N*****		
ABR17	M*****V*****DYV*****T*****PK*****L*****E*****V*****VI*****KEAQ*****I*****L*****L*****K*****NY*****LD*****AV*****F*****N*****L*****P		
ABR18	M*****Y*****ND*****T*****P*****K*****F*****V*****H*****L*****V*****V*****I*****T*****L*****V*****V*****LT*****G*****Q*****LY*****V*****A*****D*****K*****FE*****N*****		
Birch Betv 1	M*****NY*****TE*****VIP*****A*****R*****F*****FIL*****G*****NL*****VA*****QAIS*****NI*****P*****GF*****PE*****Y*****K*****DR*****V*****DEV*****HT*****FK*****N*****V*****IE*****G		
Potato STH2	M*****TS*****Y*****THE*****T*****T*****TR*****F*****LV*****V*****S*****NL*****LM*****Q*****V*****NI*****A*****D*****S*****M*****MN*****GS*****PI*****YL*****K*****HV*****DK*****V*****TK*****M*****IE*****D		
ParsleyPR1-1	M*****Q*****KS*****VE*****S*****SAEK*****F*****GL*****CL*****I*****LL*****Q*****V*****G*****AI*****S*****TL*****D*****V*****V*****LV*****HL*****GD*****AS*****PE*****TM*****K*****Q*****V*****DA*****K*****T*****FT*****ID*****D		
Celery Api g1	M*****Q*****H*****V*****LE*****L*****S*****SAEK*****IF*****Q*****GF*****VI*****V*****VL*****A*****G*****AY*****K*****D*****L*****I*****TL*****PD*****G*****P*****IT*****MT*****LR*****DG*****VN*****KE*****A*****TF*****D*****VID*****D		
Asparag. PR1	MSSGSWSHEVAVN*AGRME*AML*WHNLG*IV*FIAGGSV*S*DSV*REIKINNPAIPFSY*KERLDFV*HDKFEVKQTL*E*G		
GinsengRNase	**QKT*VEA**T*PAQK**A		KQR*DA**KDA*T*T**G*
	120	150	
BeanPR-10a	ALPETAEKITFDSKLSGPNNGSLIKLSIYHSGKDAPPNEDELKAGKAKSDSLFKAVEAYLLANP		156
PR-10b	**D*****SI*****V*****V*****K*****A*****A*****VI*****A		155
PR-10c	*****I*****V*****V*****K*****T*****A*****VI*****A		155
SoybeanSAM22	*****D*****VA*****A*****AG*****TVK*****ET*****E*****Q*****T*****A*****A*****I*****H*****D*****Y*****N		158
H4	G*****D*****V*****EC*****AA*****A*****AG*****TVK*****QT*****Q*****P*****D*****I*****V*****A*****A*****H*****D*****N		158
Pea I49	GF*****D*****V*****S*****EA*****A*****IA*****V*****K*****FT*****A*****S*****EQ*****TD*****G*****G*****C*****H*****D*****N		158
ABR17	G*****H*****SL*****VA*****ET*****I*****LA*****SD*****IV*****I*****VK*****T*****ALSDAVRDET*****GT*****G*****I*****G*****V*****G*****		157
ABR18	*ISDIV*****S*****EA*****FE*****VG*****MIV*****K*****I*****K*****VEE*****G*****A*****I*****V*****N*****N		158
Birch Betv 1	PIGD*****L*****S*****NEI*****IVAT*****D*****IL*****I*****NK*****T*****HEVKAEQ*****V*****S*****EM*****GET*****LR*****S*****H*****SD*****A*****N		160
Potato STH2	V*****G*****DK*****L*****S*****SY*****L*****FE*****AH*****G*****C*****VC*****SITE*****T*****Y*****VL*****K*****DE*****H*****NE*****K*****NQ*****G*****ME*****I*****SV*****A		155
ParsleyPR1-1	I*****L*****G*****FT*****S*****NN*****H*****FT*****AV*****PN*****AD*****CT*****V*****ST*****I*****ENT*****V*****VP*****ENI*****F*****AND*****QN*****LT*****I*****		155
Celery Api g1	I*****L*****G*****FI*****S*****EN*****HV*****V*****PT*****AD*****IC*****TT*****AI*****F*****T*****V*****VP*****ENI*****Y*****ANE*****QN*****TA*****L*****I*****		154
Asparag. PR1	G*****G*****KM*****F*****CAT*****H*****F*****EP*****SS*****C*****V*****VT*****AS*****--*****IL*****G*****VA*****SA*****KA*****EG*****IT*****NH*****M*****T*****TA*****V		158

Fig. 3. Alignment of amino acid sequences of PR-10 proteins from various plants. Amino acid sequences are deduced from cDNA or genomic clones except for the ginseng ribonuclease, for which only two peptide sequences are available. Identities to PR-10a are indicated by asterisks. Strictly conserved residues are in bold and stippled. The conserved intron position is indicated by an arrow-head above the sequences. PR-10c is deduced from the *Ypr10*c* gene. PR-10a and PR-10b were renamed from the earlier designations PvPR1 and PvPR2, respectively (Walter et al., 1990). Other proteins are presented with the designations previously published [soybean SAM22 and H4 (Crowell et al., 1992); pea I49 (Fristensky et al., 1988); ABR17,18 (Iturriaga et al., 1994); birch *Betv* I (Breiteneder et al., 1989); potato STH-2 (Matton and Brisson, 1989); parsley PR1-1 (Somssich et al., 1988); celery *Api* g 1 (Breiteneder et al., 1995); asparagus (Asparag.) PR-1 (Warner et al., 1992); ginseng ribonuclease (RNase) (Moiseyev et al., 1994)].

not only darkness but also an endogenous rhythm is involved in the regulation.

Using the *Ypr10*c*-specific probe on the various RNA samples, we have detected slightly elevated transcript levels in lower and in diseased leaves but not in dark-treated leaves (data not shown).

***Ypr10*c* and other *Ypr10* genes are responsive to defence-related stimuli.** Bean *Ypr10* genes are strongly activated by fungal elicitor (Walter et al., 1990). Reduced glutathione (GSH), which is involved in oxidative-stress responses, has been shown to mimic elicitor-mediated reactions and to cause massive induction of genes involved in pathogen defence (Wingate et al., 1988). Similarly, salicylic acid is an important modulator of plant disease resistance and a strong inducer of genes that encode PR-1 proteins (reviewed by Raskin, 1992). *Ypr10* genes seem to be only moderately induced by salicylic acid treatments, as shown previously in soybean and asparagus (Crowell et al., 1992; Warner et al., 1994), and no data are available for GSH. To analyse the effects of these two compounds on bean *Ypr10* genes, they were applied to intact leaves by feeding through the leaf stalks immersed into the inducing solution. This mode of application was chosen because direct application to wounded (sliced) leaves resulted in tissue damage and poor quality of RNA preparations. In a time course of induction, GSH (10 mM) caused a moderate increase in the levels of *Ypr10*c* transcripts and a very strong induction of total *Ypr10* transcripts (Fig. 4d). Highest concentrations of transcripts were found approximately 12 h after the onset of treatment in both cases. Salicylic acid (10 mM) applied under identical conditions caused only a minor and delayed induction of *Ypr10* genes. Only at the final time point of the experimentation period (24 h), the total amount of *Ypr10* transcripts reached a moderately elevated level (Fig. 4d). Different signalling pathways may thus be involved in the regulation by these two exogenous stimuli.

Activity of the *Ypr10*c* promoter in transgenic tobacco is regulated in an organ-specific and senescence-related manner. Due to the high complexity of *Ypr10* gene organisation, it could not be excluded that the *Ypr10*c*-specific probe hybridised to other *Ypr10* transcripts. For this reason and for high-resolution tissue-specific and cell-specific analysis of *Ypr10*c* expression, we constructed a promoter- β -glucuronidase fusion with the available promoter (2.5 kb) fused to the β -glucuronidase reporter and integrated it into the tobacco genome. We raised ten independent transformants and selected four of them with different levels of expression for more-detailed expression analysis. We selected a highly expressing transformant (PR-GUS1), two transformants with intermediate expression levels (PR-GUS12, PR-GUS13), and one lowly expressing mutant (PR-GUS4). Mature greenhouse-grown plants from the T₁ generation, at the onset of flowering or in an early flowering stage, were analysed by a fluorometric assay for extractable β -glucuronidase activity in their vegetative organs. High levels of promoter activity were found in roots, but lower leaves also contained considerable levels of β -glucuronidase activity. There was a gradient from the top to the bottom of the plant, with top leaves exhibiting only very low activity and roots containing the highest levels. Stems contained low, but detectable, levels of β -glucuronidase activity, which did not vary much between the different stem regions from top to bottom (Fig. 5).

A tendency to shift the maximal leaf levels from the lowest leaf upwards with increasing age of the plant was observed in several of the greenhouse-grown transformants (data not shown). Since the lowest leaves in older plants occasionally became prone to various infections, we analysed the effect of continued aging of plants in shoot cultures kept under sterile conditions on agar medium in glass jars. The assays were performed with one particular clonally propagated plant (PR-GUS12-2B), since there was some variation observed between different prog-

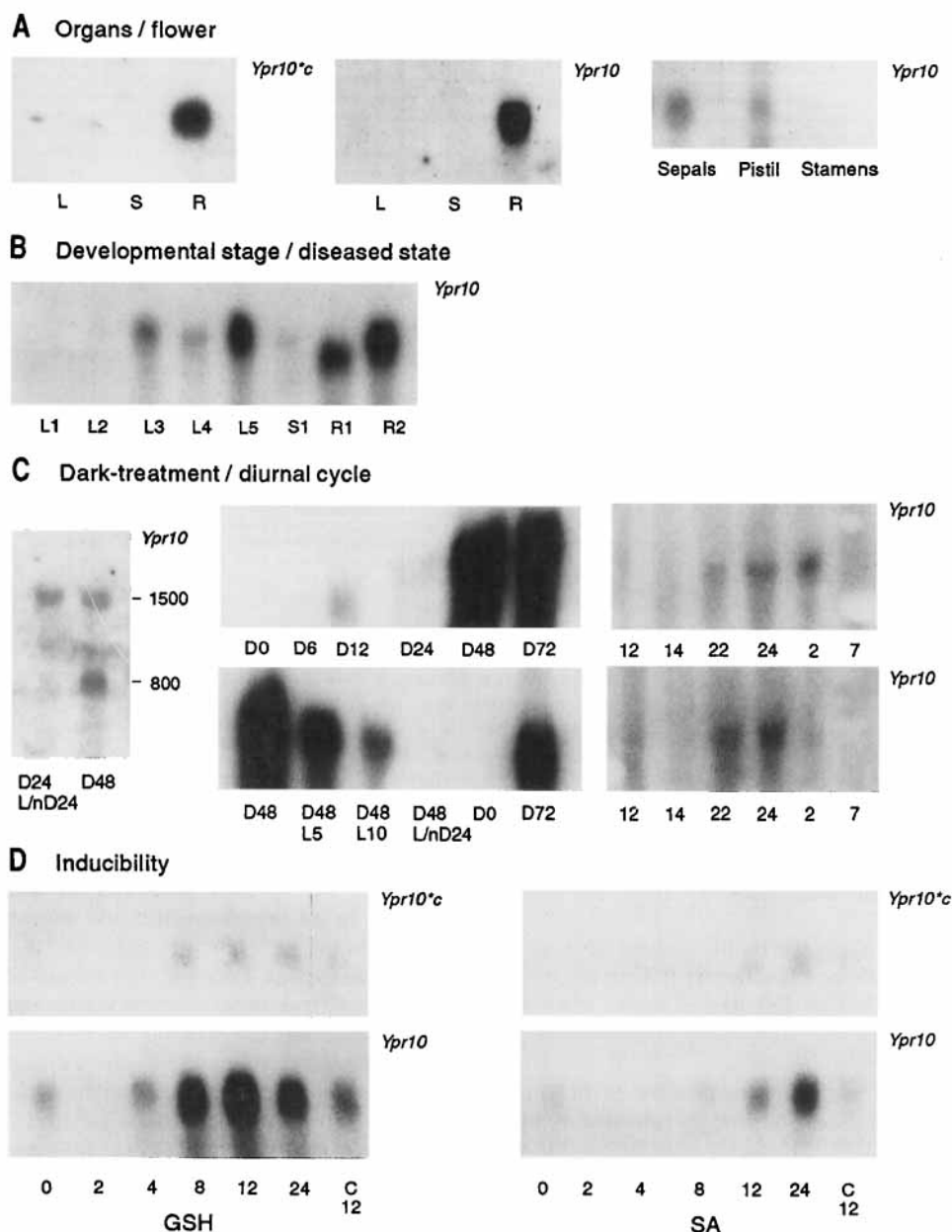


Fig. 4. mRNA levels of *Ypr10* genes in bean analysed by RNA gel blots. The probe used [gene-specific (*Ypr10*c*) or non-specific (*Ypr10*)] is indicated. The two probes gave signals for transcripts of approximately 800 bases under stringent hybridisation conditions. The blot in the left panel of (c) was probed with the *Ypr10* probe under moderate stringency. The sizes of the detected transcripts are indicated. (a) Vegetative organs and flower. Poly(A)-rich RNA from young leaves (L), mature stems (S) or roots (R) from greenhouse plants at the onset of flowering (left and center panels). Total RNA from flower parts as indicated (right panel). (b) Developmental stage and diseased state. Total RNA from mature bean plants prepared from young leaves (L1), mature leaves (L2), lower leaves (L3), lowest leaves (L4), diseased leaves (L5), young stems (S1) and lateral roots (R1, R2). (c) Effects of artificial and natural darkness. Leaf samples of total RNA from dark treatments, recovery from dark treatments and day/night-cycle experiments. Differential regulation of transcripts by darkness (left panel). Time course of regulation by dark treatment (center panel, top) or recovery from dark treatment by light (center panel, bottom). Diurnal fluctuations of transcript levels during various day or night hours in young plants (right panel, top) or mature plants (right panel, bottom). Periods of dark treatment (D), exposure to natural darkness (nD) or daylight (L) are indicated in hours. Numbers without prefixes specify the time of day, e.g. 12 = 1200, 24 = 0000. (d) Inducibility by exogenous stimuli. Time course of induction in total RNA samples by feeding GSH (10 mM) or salicylic acid (SA, 10 mM) to bean leaves. Numbers indicate the hours of treatment. C12 is a control treated with buffer only.

any plants from one particular transformant. Plants were analysed beyond the normal rejuvenation cycle of 4–6 weeks for the shoot cultures. Since the number of internodes varied between the different stages, we selected five levels, with leaf 1 corresponding to top leaves and leaf 5 corresponding to the lowest leaf, which had retained some residual green color and was not completely chlorotic. The results presented in Table 1 indicate that indeed maximal values of promoter activity change

from the lowest leaf (4 weeks of culture) to intermediate leaf levels (10–14 weeks) and further to the top leaves in very old plants. The highest values on an absolute scale were obtained at intermediate stages of senescence (e.g. on leaf level 3 after 14 weeks of culture) in leaves that appeared to be still fully green and healthy. Therefore, the onset of senescence seems to trigger *Ypr10*c* promoter activity rather than the subsequent events of breakdown of the photosynthetic apparatus.

Table 1. β -glucuronidase activity in tobacco transformant PR-GUS12-2B during senescence. Different leaf levels and roots were assayed for β -glucuronidase activity. Individual shoot cultures of a clonally propagated transgenic plant were grown on agar medium under controlled conditions. Maximal leaf values at a specific age are indicated in bold. n.d., not determined.

Tissue	β -glucuronidase activity in plants grown for							
	4 weeks	8 weeks	10 weeks	14 weeks	16 weeks	18 weeks	20 weeks	22 weeks
	pmol 4-methyl-umbelliferone $\cdot$ min ⁻¹ $\cdot$ mg protein ⁻¹							
Leaf 1	950	650	1750	400	500	2050	1800	900
Leaf 2	n.d.	1250	1900	650	1400	n.d.	n.d.	720
Leaf 3	1500	1600	2720	7500	2900	1650	600	700
Leaf 4	1700	1400	4520	6500	2600	n.d.	n.d.	670
Leaf 5	2650	2320	2420	1850	800	n.d.	n.d.	490
Roots	n.d.	2350	3960	4250	n.d.	2450	1800	2770

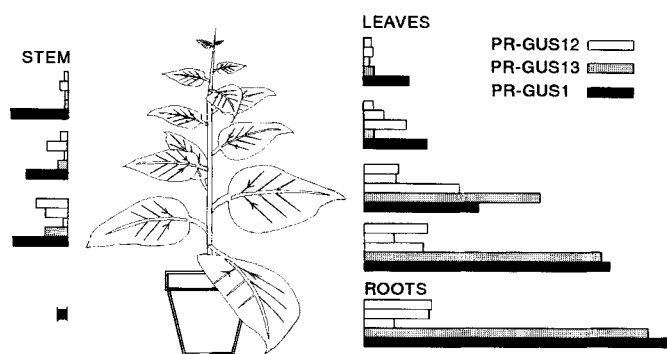


Fig. 5. Organ-specificity of *Ypr10*c* promoter activity and distribution in individual leaves and in stem regions. β -glucuronidase activity in transgenic tobacco plants that harboured a *Ypr10*c*-GUS gene fusion was determined from greenhouse-grown T₁ plants at the onset or early stages of flowering. Three different transformants (PR-GUS1, PR-GUS12 and PR-GUS13) were used. The bar represents 100 pmol 4-methyl-umbelliferone $\cdot$ min⁻¹ $\cdot$ mg protein⁻¹.

Tissue-specific localisation of *Ypr10*c* promoter activity during development. The developmentally regulated, tissue-specific expression patterns of the *Ypr10*-GUS construct in vegetative and reproductive organs were investigated by histochemical staining of transgenic tobacco plants with the X-Glu substrate. In young roots, the distribution of tissues and cells with β -glucuronidase-positive staining was quite variable. In agar-grown plantlets, we repeatedly observed enhanced expression behind the root tip, around root-branching sites and at lateral-root-initiation sites (Fig. 6a). Occasionally we detected expression in vascular tissues but usually not restricted to this tissue type. In greenhouse plants grown in soil, expression was extremely variable with various cell types apparently expressing β -glucuronidase under particular circumstances. Controls with plants that contained a promoterless β -glucuronidase gene or with wild-type SR1 tobacco plants lacked β -glucuronidase-positive blue staining, which indicated that the patchy patterns of β -glucuronidase expression in *Ypr10*c*-GUS transformants were not caused by soil bacteria. Similar experiments with roots from plants that contained cauliflower-mosaic-virus-promoter-GUS constructs showed much more continuous staining patterns, but not completely homogeneous distributions of color. Given these results, it is difficult to assign *Ypr10*c* promoter activity to a particular tissue or cell type in young roots. In the main root with strong secondary thickening, β -glucuronidase activity was localised in the developing xylem at the innermost cell layers of the mature

xylem and extended into lateral roots at lateral-root branching sites (Fig. 6b). Similarly, in young and more-mature stems, *Ypr10*c* promoter activity was seen in the developing xylem and sometimes in the developing phloem. Sites of *Ypr10*c* promoter activities in stems correlated to a considerable extent with promoter activity of a gene involved in lignification (cinnamyl-alcohol dehydrogenase), which was studied independently in tobacco transformants (Walter et al., 1994b). However, the intensity of *Ypr10*c*-GUS transformant staining was rather weak compared with the lignification-related gene promoter (data not shown). Node regions exhibited a more-intense staining than internodes, with stained areas extending into newly developing leaf stalks (Fig. 6c). In young leaves, which showed only very limited levels of β -glucuronidase activity in the fluorometric assay, a weak staining of minor veins, but not of the leaf midrib, was observed in several experiments (Fig. 6d). In senescent leaves, the staining pattern was different from that in young leaves. Strong β -glucuronidase activity accumulated preferentially in leaf blades of senescent plants (Fig. 6e). This effect was observed in shoot cultures maintained on agar medium (Fig. 6e) and in greenhouse-grown plants (Fig. 6i).

Flowers of various stages before, at and after anthesis were dissected and stained for β -glucuronidase activity. β -glucuronidase activity was absent from petals and only occasionally observed at the tip of sepals. Intense staining was seen in mature pollen at and shortly after anthesis (data not shown). Strong β -glucuronidase activity was observed specifically in the transmitting tract of styles (Fig. 6f). In contrast, the stigma and the ovary did not display β -glucuronidase activity. β -glucuronidase-positive staining in styles was strongest at anthesis in stages 11–12 of flower development as defined by Koltunow et al. (1990), but was occasionally seen to a weaker extent at earlier stages. It was most intense in the region below the stigma but sometimes extended into the lower parts of the style. Promoterless control plants did not show β -glucuronidase activity in styles and plants that contained the cauliflower-mosaic-virus-promoter-GUS construct did not show such a tissue-specific staining pattern (data not shown). Seeds stained at various stages of development did not show histochemically detectable β -glucuronidase activity (data not shown).

GSH is the strongest exogenous inducer of promoter activity.

GSH caused strong accumulation of *Ypr10*c* and other *Ypr10* transcripts when fed to bean leaves (Fig. 4d). We fed GSH, salicylic acid and two other abiotic elicitors of plant stress responses to young leaves of various *Ypr10*c*-GUS transformants grown in a greenhouse. The results of five independent experiments are summarised in Fig. 7. Stimuli were fed in a low-concentration-

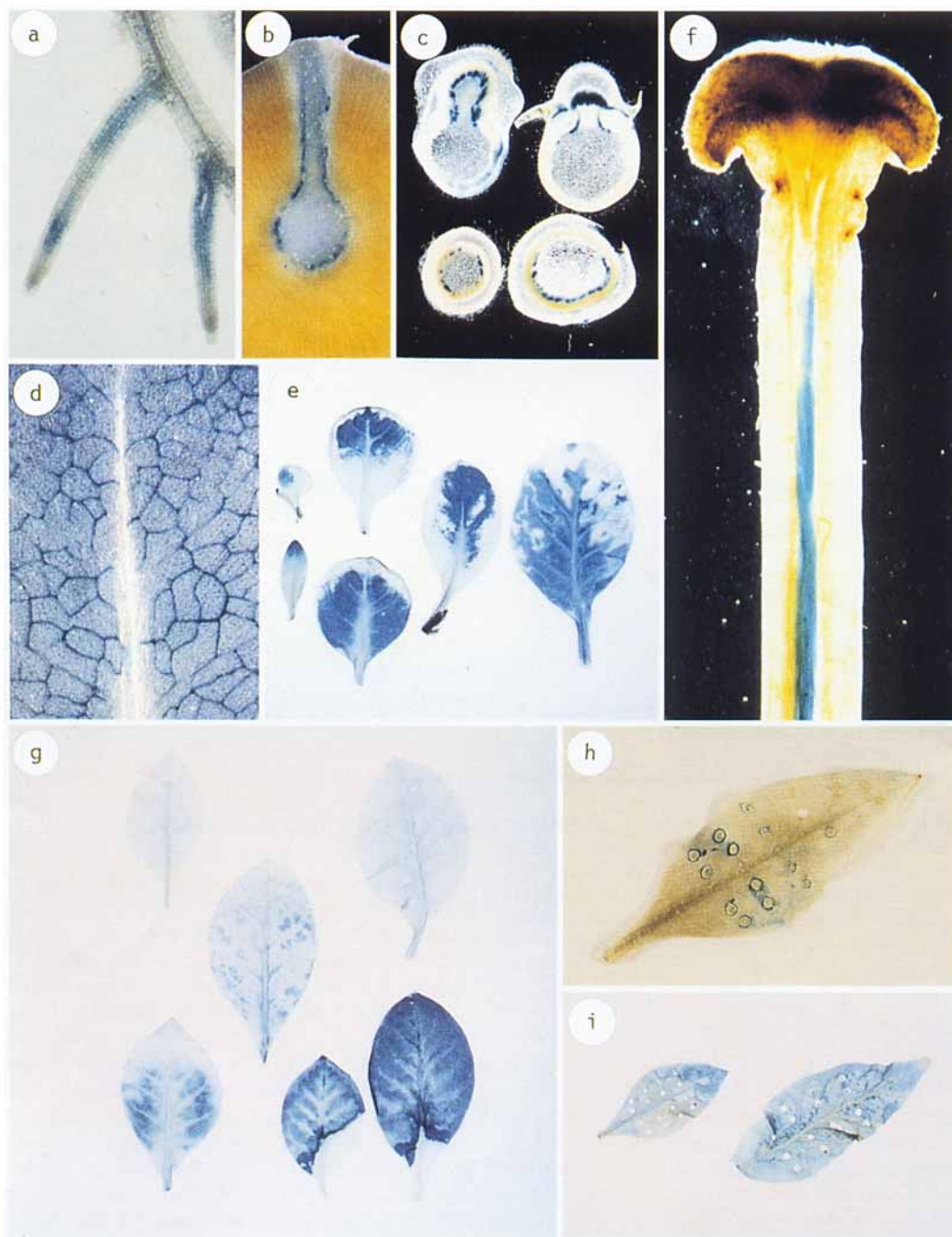


Fig. 6. Histochemical localisation of β -glucuronidase activity in transgenic tobacco plants containing the *Ypr10c-promoter–GUS fusion.** (a) Roots from a young seedling grown in agar medium, 15 $\times$ magnification; PR-GUS13. (b) Cross-section of a main root; 60 $\times$ magnification; PR-GUS1. (c) Cross-sections from node regions of young stems (top) or internodes of more mature stems (bottom), 25 $\times$ magnification; PR-GUS1 and PR-GUS12, respectively. (d) Part of very young leaf from a seedling grown in agar medium comprising midrib (center) and adjacent minor veins, 8 $\times$ magnification; PR-GUS1. (e) Collection of leaves from top (left) to bottom (right) from a senescent plant grown on agar medium for 16 weeks; PR-GUS12. (f) Stigma and style from flower at anthesis, 50 $\times$ magnification; PR-GUS12. (g) Leaves from PR-GUS12 plants grown on agar medium that were untreated (two leaves on top), fed with phosphate buffer (center), or fed with 50 μ M methyl viologen (bottom, left) or 10 mM GSH (two leaves on bottom right) for 40 h. (h) Local application of stimuli to wound sites on a young leaf of a young plant (PR-GUS12) and incubation for 40 h. Applications were performed to four sites across the leaf for each stimulus: mercuric chloride (200 μ M, sites on bottom of leaf); GSH (10 mM, adjacent sites upwards), salicylic acid (10 mM, adjacent), phosphate buffer (adjacent) and dry incubation (top of leaf). (i) Same set of exogenous stimuli except for dry incubation applied to newly developed leaves from old greenhouse-grown plants (PR-GUS1 and PR-GUS12).

phosphate buffer (pH 5.8) to provide constant pH conditions over a period of 40 h. The buffer itself caused only a slight induction of β -glucuronidase activity compared with untreated leaves. Addition of 10 mM GSH to the buffer resulted in a six-fold increase in β -glucuronidase activity and 5 mM salicylic acid

gave a threefold increase (Fig. 7). The variations in expression were quite high, in particular for the salicylic acid treatment. These variations are probably caused by toxic effects (loss of turgor and wilting symptoms) of salicylic acid treatment, which was dependent on the concentration applied. Less than 5 mM

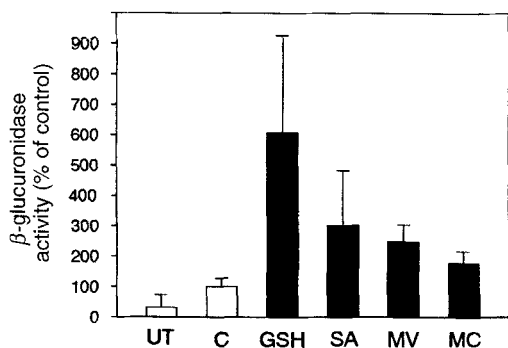


Fig. 7. Induction of β -glucuronidase activity in transgenic tobacco leaves by exogenous stimuli. Defence-related and stress-related stimuli were fed to young leaves from different transformants grown in a greenhouse through leaf stalks immersed in inducing solutions for 40 h prior to extraction for β -glucuronidase activity. Data are shown as percentages of control (phosphate buffer). Values represent the mean $\pm$ SD of five independent experiments with duplicate determinations of β -glucuronidase activity. C, control; UT, untreated; GSH, 10 mM GSH; SA, 5 mM salicylic acid; MV, 50 μ M methyl viologen; MC, 200 μ M mercuric chloride. Transformants PR-GUS1, 4 and 12 were employed in the analysis.

salicylic acid resulted in only minor effects, whereas more than 5 mM led to the described detrimental effects. Similar detrimental effects of salicylic acid on SR1 tobacco leaves have been reported previously (Warner et al., 1994). Optimal concentrations for induction are probably different for individual leaves. Methyl viologen caused a 2.5-fold induction and mercuric chloride led to a 1.8-fold increase in β -glucuronidase activity (Fig. 7). Lack of significant changes in β -glucuronidase activity in control plants exposed to these stimuli has been shown (Schaaf et al., 1995).

Since even young greenhouse transformants occasionally contained somewhat elevated endogenous levels of β -glucuronidase activity, probably caused by stress-related or infection-related factors, we also performed feeding experiments of stimuli on sterile shoot cultures, which were subsequently subjected to histochemical staining. Fig. 6 g shows the result of such an experiment with the PR-GUS12-2B clonal plant. Detachment of leaves and treatment with the buffer caused only few minor patches of β -glucuronidase expression on the leaf blade. After treatment with methyl viologen, we observed much more intense and evenly distributed β -glucuronidase activity in the leaf blade (Fig. 6 g). Addition of glutathione to the feeding buffer gave very intense and largely continuous blue staining of the leaf blade with only minor staining of the leaf veins. The part of the leaf immersed into the inducing GSH solution was not stained. If the stimuli were applied locally to wounds of a young leaf, we observed local induction around the wounded application sites (Fig. 6 h). The intensity of staining corresponded to the inducer strength, determined by the feeding assays, with minor deviations. Newly developed leaves on old greenhouse-grown plants exhibited strong endogenous β -glucuronidase activity but did not respond to the exogenous stimuli (Fig. 6i). Preliminary experiments on dark induction of PR-GUS transformants did not show significant induction by a 48-h dark treatment (data not shown).

DISCUSSION

Organisational, structural and regulatory features of genes that encode PR-10 proteins and various allergenic proteins. We investigated the organisation and regulation of a highly polymorphic *Phaseolus vulgaris* L. gene family, that encodes intra-

cellular, probably cytosolic, PR-10 proteins. In particular, we isolated and studied one member, the *Ypr10*c* gene, which was shown to contain a coding sequence very similar, but not identical, to previously isolated transcripts. These closely related transcripts have been shown to be strongly induced by fungal-elicitor treatment in bean cell cultures (Walter et al., 1990). We reinvestigated the size of the bean *Ypr10* gene family and found evidence for a larger number of *Ypr10* genes (at least 20 genes; Fig. 2) than previously estimated (Walter et al., 1990). Analysis of other plant genomes gave lower estimates for gene copy numbers (Somssich et al., 1988; Matton and Brisson, 1989; Crowell et al., 1992) but these estimates do, at least in some cases, not include additional genes, which encode PR-10 proteins but whose DNA sequence is not similar enough to cross-hybridise under the conditions used (van de Löcht et al., 1990; Iturriaga et al., 1994).

The discovery of structural similarities between PR-10 proteins and the birch-pollen allergen *Bet v* I provided evidence for a widespread occurrence of PR-10 proteins in the plant kingdom (Breiteneder et al. 1989; Walter et al., 1990). PR-10-type proteins or their genes have been characterised in a large variety of plants. Among these proteins, the birch-pollen allergen *Bet v* I and its many isoforms, and related pollen allergens in other trees are of particular interest, since they cause rhinoconjunctivitis (hayfever) in allergic humans (Breiteneder et al., 1989; Swoboda et al., 1995a). Moreover, recent reports demonstrate that *Api g* 1 and *Mal d* 1, two major food allergens of celery (*Apium graveolens*) and apple (*Malus domestica*), respectively, are members of the PR-10 class of intracellular PR proteins (Breiteneder et al., 1995; Vanek-Krebitz et al., 1995). *Api g* 1 exhibits high sequence similarity (79%) to parsley PR-10 proteins (Breiteneder et al., 1995; Fig. 3).

Concomitantly with the identification of major tree pollen, vegetable and fruit allergens as PR-10 proteins, a large number of developmental cues have been reported to regulate the expression of the defence *Ypr10* genes (Barratt and Clark, 1993; Crowell et al., 1992; Warner et al., 1993, 1994; this report). There are conflicting reports as to whether the genes expressed during development are identical to those expressed in defence against pathogens or stress. Swoboda et al. (1994, 1995b) concluded that in the birch system different genes are expressed in pollen and in cell cultures treated with fungal or bacterial pathogens. In contrast, Warner et al. (1993, 1994) showed that the AoPR-1 promoter directs expression in developing pollen and in vegetative tissues upon pathogen infection or application of defence-related stimuli. In our study, we have not investigated pathogen-induced regulation, but *Ypr10*c*-promoter-bearing plants accumulated β -glucuronidase activity in leaves after treatment with chemical elicitors of plant defence (GSH, salicylic acid) and showed promoter activity in mature pollen. We did not obtain a hybridisation signal with the RNA preparation from bean stamens. It is possible that we lost the tiny pollen grains during the preparation or that we have missed a limited period of expression in bean pollen. It is also possible that the bean promoter is inappropriately regulated in tobacco pollen. Since artefacts in pollen-specific expression of β -glucuronidase constructs are known (Uknes et al., 1993), we cannot unequivocally prove expression of bean *Ypr10* genes in pollen, although we did not observe β -glucuronidase-positive pollen from control plants (promoterless and SR1 wildtype) under our staining conditions. We and others (Constabel and Brisson, 1995; Warner et al., 1993, 1994) have shown that there is a wide range of developmental and defence-related tasks for one particular *Ypr10* gene. Not all *Ypr10* genes are universally expressed but there is some division of labour. For example, we have not observed seed-specific expression of our *Ypr10*c*-GUS construct, which has

been observed by others (Barrat and Clark, 1991; Warner et al., 1994). We found *Ypr10* transcripts in bean pistils and localised *Ypr10*c* promoter activity in tobacco pistils exclusively in the transmitting tract of styles, yet others have located it in the stigma but not in styles (Constabel and Brisson, 1995; Warner et al., 1994).

Dark treatment strongly induces one or more member(s) of the bean *Ypr10* gene family (Fig. 4). We obtained data that links *Ypr10* gene activation to the onset of senescence in leaves (Fig. 5, Table 1). A subfamily of PR-1 proteins structurally unrelated to the PR-10 class is regulated concomitantly by darkness and by senescence (Azumi and Watanabe, 1991), and transcripts for basic PR-1 proteins accumulate during natural and artificial dark periods (Eyal et al., 1992). Moreover, a timing of senescence-related expression similar to that directed by the *Ypr10*c* promoter has been described for a PR-1 protein gene (Grüner and Pfitzner, 1994). This finding implies a more general unknown physiological function for the PR-1 proteins. Such a proposal is supported by the recent identification of proteins in human brain tumors with structural similarity to tobacco PR-1 (Murphy et al., 1995) and by the existence of PR-1-related allergenic proteins in hornet and wasp venoms (Lu et al., 1993).

Regulation of plant ribonucleases and correlation with *Ypr10* gene expression. It was suggested that PR-10 proteins may function as ribonucleases based on the high sequence similarity of a ginseng ribonuclease to parsley PR-10 proteins (Moiseyev et al., 1994). Such a function would be in agreement with a proposed general role in metabolism implied by the multitude of developmental expression patterns. While there is no additional evidence for the validity of this assumption, we tried to compare available features of plant ribonucleases (reviewed by Green, 1994) with data on *Ypr10* gene regulation from different systems, including the work described in this report.

Plant ribonucleases are found in various cellular compartments. Most of the available information is on extracellular and vacuolar ribonucleases. Green (1994) states that 'knowledge about ribonucleases that reside in the cytosol is extremely limited not only in plants but also in other eukaryotes, which is unfortunate because the cytosol is presumably the major site of mRNA degradation' and that 'the vast majority of knowledge on plant RNases has so far been obtained outside the plant pathology field'. Consequently, there is no information available on specific cytosolic ribonucleases involved in plant defence.

Senescence is a major factor that affects ribonuclease activity. In flag leaves of greenhouse-grown wheat plants, three biochemically distinct RNase activities increased during the early postanthesis period and underwent further accelerated accumulation during natural senescence. Two of these activities also increased in dark-induced senescence after two days of artificial darkness and returned to normal levels after light-induced recovery (Blank and McKeon, 1991). Senescence-associated expression of *Ypr10* genes has been shown by Crowell et al. (1992). We corroborated this finding and provided evidence for a specific *Ypr10* gene activity in the leaf blade during senescence (Fig. 6e). By measurement of promoter activity during the progression of senescence we found highest activities at the onset of senescence before visible symptoms of disassembly of the photosynthetic apparatus appeared (Table 1). Dark-induced accumulation, with similar kinetics to those described for wheat leaf ribonucleases, and a diurnal rhythm of elevated transcript levels during the night, as shown here (Fig. 4c) and briefly discussed above, are characteristics of *Ypr10* genes described in this report.

The discovery that the S-glycoproteins involved in gametophytic self-incompatibility are ribonucleases (McClure et al.,

1989) and that they are essential for the rejection of self-pollen (Huang et al., 1994) has provided a major stimulus to the study of plant ribonucleases and provided support for the idea of a large diversity of functions. Several studies (summarised by Green, 1994) have demonstrated high levels of expression of S-ribonuclease genes in the transmitting tract of the style (particularly the upper portion). We have observed such a phenomenon for one particular member (*Ypr10*c*) of the bean *Ypr10* gene family by a promoter-activity assay (Fig. 6f). The role of the S-ribonucleases in pistils seems to involve more than the self-incompatibility mechanism, since *Petunia inflata* contains other structurally distinct S-ribonucleases in this tissue, which are unlinked to the S-locus (Lee et al., 1992). The S-like ribonuclease (*RNS2*) gene of *Arabidopsis* is expressed in pistils (stigma and style) and petals and during senescence (Taylor et al., 1993). Petal-specific and stigma-specific expression are also reported features of *Ypr10* genes (Constabel and Brisson, 1995; Warner et al., 1994) and may be complemented by additional genes for style-specific expression such as the bean *Ypr10*c* gene (Fig. 6 f).

Perhaps the most striking piece of circumstantial evidence for a ribonuclease function of PR-10 proteins is related to their abundance in pollen and their action as tree-pollen allergens. The major grass-pollen allergens (*Phl p* Vb) that cause allergic rhinoconjunctivitis or bronchial asthma (which are also caused by the structurally unrelated PR-10 proteins) have been identified as ribonucleases (Bufe et al., 1995). The grass-pollen ribonucleases share another feature with PR-10 proteins, that is, accumulation of an immunologically cross-reactive species upon salicylic acid treatment of a tissue (inflorescence) where they are not normally expressed. Based on this finding, a role of *Phl p* Vb in host-pathogen interactions of pollen has been proposed (Bufe et al., 1995).

Possible functional roles of PR-10 proteins in development and defence. The evolutionary conservation and the widespread distribution of expression of *Ypr10* genes in plant development suggests a general role in plant metabolism and not a mere inhibitory action on pathogen spread. This proposal is consistent with the finding that birch-pollen washes, which contain high amounts of the *Bet v* I allergen protein, do not display antimicrobial activity (Swoboda et al., 1994). What might be the physiological role of PR-10 proteins if we consider the present hypothesis of ribonuclease function and take into account that nucleolytic activities can range from degradation of single RNA and DNA species to autolysis of whole cells?

One major determinant in the control of gene expression, which is often not considered, is the rate of mRNA degradation. Current models favor parameters inherent to the mRNA itself such as cap structures or poly(A)-tail structures as major regulatory factors for mRNA destabilisation (Sachs, 1993). Destabilisation of mRNAs is an active and selective process, which requires newly synthesized compounds and specific binding to particular sites in an mRNA (Fritz et al., 1991; Zhang et al., 1993; Zhang and Mehdy, 1994). In the heat-shock responses, selective degradation of existing mRNAs has been shown to assist in the rapid restructuring of the cellular pattern of protein synthesis (Belanger et al., 1986; Brodl and Ho, 1991). A potential physiological function of cytosolic ribonucleases, as suspected for PR-10 proteins, could therefore be an involvement in selective or highly regulated degradation of existing mRNAs during stress or pathogen attack rather than wholesale degradation of cellular RNAs.

Pathogen responses in animal systems are often associated with nucleolytic activities, which eventually lead to programmed cell death (Collins and Rivas, 1993). In plants, several individual

ribonuclease activities were elevated in rust-infected wheat leaves (Barna et al., 1989). Increases in ribonuclease and protease activities have been shown in tobacco leaves that exhibit a local programmed cell death during the hypersensitive response to tobacco mosaic virus (Lusso and Kuć, 1995). A direct link between plant cells that exhibit a hypersensitive response and *Ypr10* expression is lacking, but it has been shown that *Ypr10* transcripts are induced locally around wounds, infection sites or exogenous-stimulus-application sites (Fig. 6 h; Schmelzer et al., 1989; Somssich et al., 1988; Warner et al., 1993). Race-specific accumulation of PR-10 proteins after inoculation with low concentrations of *Phytophthora infestans* has been reported (Constabel and Brisson, 1992). Recently, a system of engineered programmed cell death that leads to fungal resistance in potato plants, which uses a bacterial ribonuclease driven by a fragment of an infection-specific plant promoter, has been established (Strittmatter et al., 1995). The feasibility of such a system suggests that plants may have developed similar endogenous, but probably more subtle, strategies of cytotoxic ribonuclease expression in the hypersensitive response.

Aspects of developmentally programmed cell death occur during vascular development and xylogenesis, which involve autolysis of cells. Several ribonucleases are induced in *Zinnia elegans* cells differentiated synchronously to xylem cells by hormonal treatment. A 17-kDa ribonuclease activity is particularly interesting, since its activity is induced transiently in a fairly early stage of xylem differentiation (Thelen and Northcote, 1989). This ribonuclease activity might perhaps have a spatial and temporal relationship to the *Ypr10* promoter activity in the vascular tissue of leaves, stems and roots (Fig. 6 b–d). The similarity of spatial transcriptional activity in developing xylem and phloem of stems between the *Ypr10**c promoter and a cinnamyl-alcohol-dehydrogenase promoter, which drives a gene of lignin biosynthesis, is consistent with such an explanation (Fig. 6 c; Walter et al., 1994b). This hypothesis is further supported by the results of Warner et al. (1994), who showed that the pattern of transcriptional activity of an AoPR1-GUS fusion was very similar to that of stem lignification.

While these models may provide the basis for new long-term experimental approaches, it is now imperative to demonstrate *in vitro* a ribonuclease activity of PR-10 proteins (if any) and to determine the preferred RNA substrate. Preliminary experiments failed to prove such a ribonuclease activity (Constabel and Brisson, 1995; Warner, S. and Draper, J., personal communication). We have initiated additional such experiments but conclusive results are not available.

In conclusion, there is no final proof or disproof for the hypothesis of a ribonuclease function for PR-10 proteins. However, our contribution of circumstantial evidence towards such a function should encourage further investigations into this topic.

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