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Structure and expression of genes coding for structural proteins of the plant cell wall

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CONTENTS

SUMMARY

The best-known protein components of the plant cell wall have highly repetitive, proline-rich sequences. The use of recombinant DNA approaches has enabled complete sequences of these proteins to be determined and features of the expression of the corresponding genes to be examined. These results, coupled with the use of immunological techniques, have shown that proline-rich proteins are interesting probes to study developmental and defence processes in plants. In this review, the sequence and expression of different groups of proline-rich proteins in plants are presented. These groups include hydroxyproline-rich glycoproteins (HRGP) or extensins, proline-rich proteins (PRP) and glycine-rich proteins (GRP). The specific features of each group and the possible functions of these proteins are discussed, as well as the data available on the mechanisms controlling the expression of their corresponding genes.

Key words: Cell wall proteins, hydroxyproline-rich glycoproteins, proline-rich proteins, glycine-rich proteins.

I. INTRODUCTION

The cell wall is one of the most characteristic structures of plant cells. In the absence of movement, the definition of the new wall is an essential step in plant morphogenesis, and wall elongation is the main mechanism of plant cell growth. Plant cells communicate with the environment and with the other cells through structures in the wall. The cell wall is also an essential element of plant protection against the attack of pathogens or stress conditions. Plant viruses, for instance, have to find ways to pass this barrier and to use special proteins – the movement proteins – to allow them to cross the walls through plasmodesmata (Ding *et al.*, 1992). For all these reasons, cell wall components have been studied at morphological and biochemical levels. Complex components such as lignins, polysaccharides or waxes have been described. Proteins having enzymatic or structural functions have also been characterized. Hundreds, probably thousands, of genes act upon the formation and dynamics of the plant cell wall (see Roberts, 1989, 1990; Varner & Lin, 1989; Bowles, 1990 for recent reviews).

The dynamics of the cell wall have an obvious essential role in cell development. Its components must interact with the elements that define the timing and the spatial distribution of synthesis and

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Table 1. *cDNA and genomic clones encoding cell wall hydroxyproline-rich glycoproteins*

Name	Nature	Plant	aa	References
DC 5	Partial cDNA	Wounded carrot roots	43	Chen & Varner (1985*a*)
DC 5A1	Genomic	Carrot	**306**	Chen & Varner (1985*b*)
Tom-17-1	Partial cDNA	Wounded tomato stems	129	Showalter & Rumeau (1990)*
Tom J-2	Partial cDNA	Unwounded tomato stems	80	Showalter & Rumeau (1990)*
Tom 5	Genomic	Tomato	**371**	Showalter *et al.* (1985)
Class I-UG-18	Partial cDNA	Unwounded tomato stems	nd	Showalter *et al.* (1991)
Class I-UG	Partial cDNA	Unwounded tomato stems	nd	Showalter *et al.* (1991)
Class I-W17-1	Partial cDNA	Wounded tomato stems	132	Showalter *et al.* (1991)
Class I-WY	Partial cDNA	Wounded tomato stems	67	Showalter *et al.* (1991)
Class I-W6	Partial cDNA	Wounded tomato stems	90	Showalter *et al.* (1991)
Class I-Tom J-10	Genomic	Tomato	**388**	Zhou *et al.* (1992)
Class II-uJ-2	Partial cDNA	Unwounded tomato stems	82	Showalter *et al.* (1991)
Class II-u1	Partial cDNA	Unwounded tomato stems	75	Showalter *et al.* (1991)
Class II-u2	Partial cDNA	Unwounded tomato stems	75	Showalter *et al.* (1991)
Class II-Tom-L4	Genomic	Tomato	**322**	Zhou *et al.* (1992)
CNT 1	Complete cDNA	$Transgenic_{T\text{-}Cyt}$ tobacco shoot	**318**	Memelink *et al.* (1987)
npExt	Genomic	Tobacco	**416**	De Loose *et al.* (1991)
$HRGP_{nt}3$	Genomic	Tobacco	**620**	Keller & Lamb (1989)
NaPRP 3	Partial cDNA	Tobacco mature styles	139	Chen *et al.* (1992)
NaPRP 3g12	Genomic	Tobacco	**151**	Chen *et al.* (1992)
NaClass I	Partial cDNA	Tobacco stigma/style	nd	De S. Goldman *et al.* (1992)
NaClass II	Partial cDNA	Tobacco stigma/style	nd	De S. Goldman *et al.* (1992)
NaClass III	Partial cDNA	Tobacco stigma/style	426	De S. Goldman *et al.* (1992)
Hyp 2.13	Partial cDNA	Elicitor-treated bean cells	368	Corbin *et al.* (1987)
Hyp 3.6	Partial cDNA	Elicitor-treated bean cells	163	Corbin *et al.* (1987)
Hyp 4.1	Complete cDNA	Elicitor-treated bean cells	**230**	Corbin *et al.* (1987)
HaGX 3	Genomic	Sunflower	**262**	Adams *et al.* (1992)
CW 6	Partial cDNA	Petunia callus	92	Showalter & Rumeau (1990)*
CW 7	Partial cDNA	Petunia callus	138	Showalter & Rumeau (1990)*
ptl 1	Partial cDNA	*Antirrhinum* pistil 1 d before anthesis	117	Baldwin *et al.* (1992)
ExtA	Genomic	Oilseed rape	**299**	Evans *et al.* (1990)
PRP_t566	Partial cDNA	Oilseed rape roots	134	Evans *et al.* (1990)
PRP_t999	Partial cDNA	Oilseed rape roots	227	Evans *et al.* (1990)
PRP_t1449	Partial cDNA	Oilseed rape roots	123	Evans *et al.* (1990)
PRP_t592	Partial cDNA	Oilseed rape roots	176	Evans *et al.* (1990)
PRP_t1214	Partial cDNA	Oilseed rape roots	nd	Evans *et al.* (1990)
aHRGP	Genomic	*Arabidopsis*	nd	Showalter & Varner (1989)*
paHRGP	Complete cDNA	Almond roots	**278**	Garcia-Mas *et al.* (1992)
crHRGP	Genomic	*Chlamydomonas reinhardtii*	**202**	Woessner & Goodenough (1989)
ISG	Genomic	*Volvox carteri*	**464**	Ertl *et al.* (1992)
MC 56	Partial cDNA	Maize coleoptiles (W64A)	267	Stiefel *et al.* (1988)
zmHRGP	Genomic	Maize (AC1503)	**328**	Stiefel *et al.* (1990)
zmHRGP	Genomic	Maize (W22)	**303**	Raz *et al.* (1992)
zdHRGP	Genomic	Teosinte	**350**	Raz *et al.* (1992)
svHRGP	Genomic	Sorghum	**283**	Raz *et al.* (1991)
osHRGP	Genomic	Rice	**369**	Caelles *et al.* (1992)

aa, Number of amino acids including the signal peptide; complete sequences are indicated in bold figures. nd, not determined.

* Cited by these authors.

deposition of new materials in the growing cell, in particular the cytoskeleton. This interaction is complex, and feedback effects have been shown. For instance, carbohydrate oligomers have been shown to act upon morphogenic pathways (Eberhard *et al.*, 1989; Marfà *et al.*, 1991), indicating that the control enzymes related to metabolism of cell wall components may play an essential role in the regulation of plant developmental processes. In this sense, the study of the function of enzymes linked to the formation and degradation of cell wall may, in the near future, be the source of interesting information regarding plant cell dynamics. The carbohydrate component of the plant cell wall has also been studied by using specific monoclonal antibodies raised against plant protoplasts. In this way the presence of specific epitopes during plant cell development, which in some cases are position-

dependent instead of cell-type dependent, has been demonstrated (Knox, Day & Roberts, 1989). In the same direction, genes, whose mutations arrest embryogenesis at defined stages of development, code in some cases for proteins that are secreted and that may have a role in the plant cell wall (Cordewener *et al.*, 1991). All these data confirm the importance of genes that take part in wall formation for specific plant cell functions related to development.

The application of molecular biological methodologies has allowed a very rapid increase of our knowledge on the structure and expression of some of the main proteins that form the cell walls of a number of species. The best-known components are those corresponding to the most abundant structural proteins, mainly hydroxyproline-rich and glycine-rich proteins.

II. HYDROXYPROLINE-RICH GLYCOPROTEINS (HRGPS)

1. *Occurrence and structure of HRGP cell wall proteins*

The analysis of the proteins present in plant cell walls produced a high proportion of hydroxyproline as the major amino acid constituent in cell wall hydrolysates (Lamport & Northcote, 1960). Three main classes of glycoproteins containing hydroxyproline in plants have been described: lectins (restricted to the Solanaceae family), arabinogalactan proteins (AGPs) and extensins. Lectins and AGPs are soluble wall components extracted with salts while extensins are insoluble, being tightly associated with the cell wall (Showalter & Varner, 1989). The term 'HGRP' (hydroxyproline-rich glycoprotein) is normally associated in dicotyledonous species with extensins, a name that followed the assumption that they could be involved in a cell wall extensibility (Lamport, 1963). HRGPs are the most important supplier of proline and hydroxyproline to the wall (Cassab & Varner, 1988).

Extensins have been the object of a number of reviews (McNeil *et al.*, 1984; Fry, 1986; Cooper *et al.*, 1987; Tierney & Varner, 1987; Cassab & Varner, 1988; Cooper, 1988; Varner & Lin, 1989; Showalter & Rumeau, 1990). Extensin genes have been cloned (Table 1) and proteins purified (Table 2) from different plants. The study of extensins was difficult at the beginning because of their insolubility. The hydrolysis of cells walls from tomato with acid/protease treatment allowed the characterization of the resulting glycopeptide fragments that went into solution (Lamport, 1977). These glycopeptides contained arabinose, galactose, hydroxyproline and other amino acids such as valine, serine, threonine, lysine and tyrosine, and an unusual tyrosine derivative, isodityrosine, composed of two tyrosyl units cross-linked by a diphenyl ether bridge (Lamport, 1967, 1969; Lamport, Katona & Roering, 1973; Fry, 1982). It was proposed that *in vivo* the isodityrosine reaction could be catalyzed by a peroxidase or similar enzyme. Evidence for the presence of this type of cross-link *in vivo* to explain extensin insolubility in plant cell walls came from the ability of $NaClO_2$ to split isodityrosine residues in cell walls following extensin solubilization (Fry, 1982), and from the isolation of isodityrosine associated with tomato tryptic peptides from partly hydrolyzed cell walls (Epstein & Lamport, 1984). The products of hydrolysis abundantly contained the sequence Ser-Hyp_4 (Hyp is used in this article as the three-letter symbol for hydroxyproline), which has later been shown to be one of the most common repetitive elements of dicot HRGP sequences.

In 1969, a salt-extractable hydroxyproline-containing protein was identified in carrot roots by Chrispeels (1969). It was suggested that it might be the precursor of the covalently bound cell wall extensin. Extensin synthesis and secretion in carrot roots was enhanced by slicing and aeration of the tissue (Chrispeels, Sadava & Cho, 1974). In wounded carrot roots this cell wall polymer was synthesized by the sequential translation of extensin mRNA on rough endoplasmic reticulum, hydroxylation of peptidyl proline by a prolyl hydroxylase, glycosylation of hydroxyproline by oligo-arabinosides and of serine by galactose in the Golgi apparatus, and secretion into the cell wall (Chrispeels, 1970; Chrispeels *et al.*, 1974; Sadava & Chrispeels, 1978) where it would be insolubilized by a covalent link as the isodityrosine bridges (Cassab & Varner, 1988).

Extensin was first purified and analyzed by Stuart & Varner (1980). The composition of the carrot glycoprotein was similar to the composition of the insoluble extensin peptides from tomato walls studied by Lamport (1977). Since then several laboratories have purified precursors to the extensin network from different plants, tissues, callus and cellular suspensions, such as potato tuber (Leach, Cantrell & Sequeira, 1982), tobacco callus (Mellon & Helgeson, 1982), tomato cell suspension cultures (Smith, Muldoon & Lamport, 1984; Smith *et al.*, 1986), soybean seed coats (Cassab *et al.*, 1985), cucumber and sycamore-maple suspension culture cells (Heckman, Terhune & Lamport, 1988), and melon callus (Mazau, Rumeau & Esquerré-Tugayé, 1988) in dicotyledonous species. In most of these species the protein contained a high proportion of serine and hydroxyproline, related to the Ser-Hyp_4 sequence. Related proteins have been described in monocots, for instance in maize pericarps (Hood, Shen & Varner, 1988) and cell suspensions cultures (Kieliszewski & Lamport, 1987; Kieliszewski, Leykam & Lamport 1990). In gymnosperms, two proteins were purified from Douglas fir (Kieliszewski *et al.*, 1992; Fong *et al.*, 1992), and one extensin-like protein from pine (Bao, O'Malley & Sederoff, 1992).

Table 2. *Amino acid sequence repeats in HRGPs*

Group	Repeat	Number of repeats (name, plant)
0	SPPPP	10 (ISG, *Volvox*); 26 (HRGP nt3, tobacco); 6 (NaPRP 3g12, tobacco); 5 (NaClass I, tobacco); 1 (NaClass II, tobacco); 5 (NaClass III, tobacco); 14 (paHRGP, almond); 17 (HaGX3, sunflower); p (SP2, Douglas fir)[a]
	$S(P)_n$	2 (NaPRP 3g12, tobacco); nd (NaClass II, tobacco); nd (NaClass III, tobacco); nd (HaGX3, sunflower); 10 (ISG, *Volvox*)
	XPPP	8 (crHRGP, *Chlamydomonas*); 2 (ptl 1, *Antirrhinum*)
	KPP	2 (ptl 1, *Antirrhinum*)
	$(SP)_n$	16 (crHRGP, *Chlamydomonas*); 8 (NaPRP 3g12, tobacco); 9 (ISG, *Volvox*)
A	SOOOOSOSOOOOYYYK	p (cell wall peptide, tomato)[b]
	SPPPPSPSPPPPYYYK	3 (Tom 17-1, tomato); 6 (Hyp 2.13, bean); 4 (Hyp 3.6, bean); nd (uG-18 Class I tomato); nd (w17-1 Class I, tomato); nd (wY Class I, tomato)
	SPPPPSPSPPPPYY/VYK	nd (w6 Class I, tomato)
	SPPPPSPSPPPPYYY	3 (CW 7, petunia)
	$SPPPPSPSPPPPTY_{1-3}S$	nd (uJ-2 Class II, tomato)
	$SSPPPPSPSPPPPTY_{1-3}$	2 (Tom L-4 Class II, tomato)
	SPPPPSPSPPPP	2 (Tom J-2, tomato)
	SOOOOSOK	p (cell wall peptide, tomato)[b]
	SPPPPKHSPPPPYYYH	11 (Hyp 4.1, bean)
	HAPP	4 ($HRGP_{nt}3$, tobacco)
	SPPPPSPKYVYK	19 (Tom J-10 Class I, tomato)
	SPPPPYYYKSPPPPSP	8 (Tom J-10 Class I, tomato)
	SPPPPYYYK/S	nd (uG Class I, tomato)
	PYHYK	11 (paHRGP, almond)
	SP_{4-5}TPSYEHP	nd (u1 Class II, tomato); nd (u2, Class II tomato)
	SP_{2-5}TPSYEHPKTP	4 (Tom L-4 Class II, tomato)
B	SOOOOTOVYK	p (cell wall peptide, tomato)[b]; p (P1 extensin peptide, tomato)[c]
	SPPPPTPVYKYK	7 (DC 5A1, carrot)
	SPPPPTPVYK	6 (CNT 1, tobacco); 11 (npExt, tobacco); 1 (CW 6, petunia)
	SPPPPTPIYK	1 (CW 6, petunia)
	SOOOO [VKPYHP]TOVTK	p (P1 extensin peptide, tomato)[c]
	SOO [VHE/KYP]OOTOVYK	p (P1 extensin peptide, sugar beet)[d]
	LPP [DTDPAD]PP	1 (ptl 1, *Antirrhinum*)
	SOOOOVYKYK	p (cell wall peptide, tomato)[b]; p (P2 extensin peptide, tomato)[c]
	SPPPPVYK	4 (extA, oilseed rape); nd (PRP_t566, oilseed rape); nd (PRP_t999, oilseed rape); nd (PRP_t1449, oilseed rape)
	OOVYK	p (dfPHRGP, Douglas fir)[e]
	PPXXK	p (dfPHRGP, Douglas fir)[e]
	VYKSPPPP	12 (HaGX 3, sunflower)
	SPPPPVYH	8 (extA, oilseed rape); nd (PRP_t592, oilseed rape); nd (PRP_t566, oilseed rape); nd (PRP_t999, oilseed rape); nd (PRP_t1499, oilseed rape)
	VHKSPPPP	5 (HaGX 3, sunflower)
	SPPPPVH	21 (Tom 5, tomato); nd (aHRGP, *Arabidopsis*)
	SPPPPVA	8 (Tom 5, tomato); nd (aHRGP, *Arabidopsis*)
	SPPPPVKHY	nd (PRP_t592, oilseed rape)
C	SPPPPKKPYYPPHTPVYK	8 (CNT 1, tobacco)
	SPPPPKKPY/HYPPHTPVYK	6 (npExt, tobacco)
	SPPPPK/VKPYHPSPTPYHPS/APVYK	5 (npExt, tobacco)
	SPPPPKKPYHPSPTPY	1 (CW 6, petunia)
	SOOOOK	p (cell wall peptide, tomato)[b]; p (cell wall peptide, melon)[f]
	SPPPPKKXYEYK	nd (PRP_t592, oilseed rape)
	SPPPPKKHYEYK	7 (extA, oilseed rape); nd (PRP_t566, oilseed rape); nd (PRP_t999, oilseed rape); nd (PRP_t1449, oilseed rape)
	HHYKYK	4 (DC 5A1, carrot)
	SPPPPKH	7 (DC 5A1, carrot)
	SPSPPKH	4 (paHRGP, almond)
D	PPTYTP	13 (MC 56, maize W 64A); 15 (zmHRGP, maize AC 1503); 13 (zmHRGP, maize W 22); 15 (zdHRGP, teosinte); 4 (svHRGP, sorghum); 3 (osHRGP, rice)
	PPTYKP	11 (osHRGP, rice)

Table 2. *continued*

Group Repeat	Number of repeats (name, plant)
SPKPP	12 (MC 56, maize W 64A); 9 (zmHRGP, maize AC 1503); 8 (zmHRGP, maize W 22); 10 (zdHRGP, teosinte); 4 (svHRGP, sorghum)
TPKPT	12 (MC 56, maize W 64A); 11 (zmHRGP, maize AC 1503); 9 (zmHRGP, maize W 22); 11 (zdHRGP teosinte); 1 (svHRGP, sorghum)
ATKPP	2 (MC 56, maize W 64A); 1 (zmHRGP, maize AC 1503); 2 (zmHRGP, maize W 22); 3 (zdHRGP teosinte); 3 (svHRGP, sorghum)
QPKPT/NP	9 (osHRGP, rice)

A, Alanine; D, aspartic acid; E, glutamic acid; H, histidine; I, isoleucine; K, lysine; L, leucine; N, asparagine; O, hydroxyproline; P, proline; Q, glutamine; S, serine; T, threonine; V, valine; X, any amino acid; Y, tyrosine; nd, not determined; p, data obtained from purified protein. Amino acids that are equally frequent in a position are linked by a slash (/).

[a] Fong *et al.* (1992); [b] Lamport (1977); [c] Smith *et al.* (1986); [d] Li *et al.* (1990); [e] Kieliszewski *et al.* (1992); [f] Esquerré-Tugayé & Lamport (1979). References for cDNA and genomic clones are given in Table 1.

In algae, the repetitive proline-rich inversion-specific glycoprotein (ISG) (Ertl *et al.*, 1992) from *Volvox* embryos has been described. Two HRGP proteins that lack a perfect Ser-Hyp_4 motif have also been described. The first one is an HRGP isolated from sugar beet (*Beta vulgaris*) cell suspension cultures (Li, Kieliszewski & Lamport, 1990) which has an insertion sequence inside the pentamer Ser-Hyp_4 producing the SOOXOOTOVYK repeat (O is the one-letter symbol for hydroxyproline) where X = (VHE/KYP). The second one is an HRGP-like protein isolated from a gymnosperm, Douglas fir (Kieliszewski *et al.*, 1992), that has been shown to be glycosylated, poor in serine and repeats to be similar to the ones described for PRPs (see later).

Most of the HRGPs purified so far have been shown to be highly basic molecules, abundant in lysine, poor in aspartate and glutamate, and containing hydroxyproline and arabinose. Most of the Hyp is found in Ser-Hyp_4 peptide sequences. The proportion of Hyp glycosylation varies between different species. In dicotyledonous species the carbohydrate content of these proteins may be higher than 60% with hydroxyproline residues glycosylated with tri- and tetra-arabinosides (Lamport & Miller, 1971) and many of the serine residues modified with galactose (Lamport *et al.*, 1973), while in monocots only 30% of Hyp are glycosylated, mainly as Hyp-Ara_3 (McNeil *et al.*, 1984).

The secondary structure of extensin from carrot roots and tomato cells was studied by circular dichroism (Van Holst & Varner, 1984). The spectra showed that extensin is completely folded in the polyproline II conformation (an extended left-handed helix). If extensin is deglycosylated, much of the conformation is lost suggesting that the carbohydrate moiety of this glycoprotein serves to stabilize this helical conformation, presumably by intramolecular hydrogen bonding. These results were confirmed by electron microscopy of glycoprotein preparations where it appears as a rod-like structure. This structure is also lost when the deglycosylated protein is observed, appearing as an amorphous globular mass (Stafstrom & Staehelin, 1986*a*, *b*; Heckman *et al.*, 1988). Interestingly, a folded structure was also found in synthetic peptides containing a proline-rich repetitive sequence of a maize storage protein, γ-zein, provided that a certain degree of polymerization was attained (Rabanal *et al.*, 1922). One particular case is the ISG protein from *Volvox*. Electron micrographs of this protein suggest that it may contain a globular domain attached to a rod-like element (Ertl *et al.*, 1992).

Amino acid sequences of two different extensin monomers from tomato cell suspension cultures (see Table 2) indicated for the first time the presence of different extensin monomers not only in different tissues but also in the same one. It was proposed by Smith *et al.* (1984) that differences in the repeats could result from proteins having different functions in the wall network, or from genes responding to different stimuli, as has later been confirmed in different examples using DNA probes. The sequences of the peptides isolated by tryptic digestion of the isolated tomato proteins P1 and P2 (Table 2) seem to indicate a bifunctional domain in each repeat. The first domain in the repeat is glycosylated and rigid, the second non-glycosylated and flexible (Smith *et al.*, 1986). *Volvox* extensin also presents two domains at the protein level. When *Volvox* extensin sequence is analyzed, the N-terminal domain gives a probability for a globular protein conformation, while Ser-Hyp_4 repeats are restricted to the C-terminal domain of the protein. This particular structure probably explains the electron microscope image of this protein described above (Ertl *et al.*, 1992).

Although protein purification is the only way to determine the post-translational modifications which these molecules undergo (including hydroxylation of

proline residues, arabinosylation, galactosylation, and formation of diphenylether links between tyrosine residues), to obtain the sequence of the proteins is not an easy task in molecules that are difficult to extract, that are complex mixtures of polypeptides and that have repetitive sequences, as is the case for HRGPs. Therefore only partial sequences have so far been obtained by protein purification and sequencing. Complete sequences of HRGPs could not be obtained before the use of recombinant DNA techniques. Different complete and partial cDNAs have been isolated (Table 1) from wounded carrot roots (Chen & Varner, 1985*a*), wounded and non-wounded tomato stems (Showalter *et al.*, 1985; Showalter & Rumeau, 1990; Showalter *et al.*, 1991), transgenic tobacco shoots having an increased synthesis of cytokinins (Memelink, Hoge & Schilperoort, 1987), tobacco (Chen, Cornish & Clarke, 1992; de S. Goldman *et al.*, 1992) and *Antirrhinum* (Baldwin, Coen & Dickinson, 1992) mature styles, elicitor-treated bean cells (Corbin, Sauer & Lamb, 1987), petunia calli (Hironako unpublished, cited by Showalter & Rumeau, 1990), oilseed rape roots (Evans *et al.*, 1990), almond tree roots (Garcia-Mas *et al.*, 1992) and maize coleoptiles (Stiefel *et al.*, 1988). Moreover, genomic sequences (Table 1) are available from carrot (Chen & Varner, 1985*b*), tomato (Showalter *et al.*, 1985; Zhou, Rumeau & Showalter, 1992), tobacco (Keller & Lamb, 1989; de Loose *et al.*, 1991; Chen *et al.*, 1992), oilseed rape (Evans *et al.*, 1990), sunflower (Adams *et al.*, 1992) and *Arabidopsis* (Herrera-Estrella unpublished, cited by Showalter & Varner, 1989) in dicotyledonous species; maize (Stiefel *et al.*, 1990), teosinte (Raz *et al.*, 1992), sorghum (Raz *et al.*, 1991) and rice (Caelles, Delseny & Puigdomènech, 1992) in monocotyledonous species; *Volvox* ISG (Ertl *et al.*, 1992), a gene that contains introns in the coding region, and *Chlamydomonas reinhardtii* Class IV protein (Woessner & Goodenough, 1989) in algae.

Extensin genes do not have introns in their coding regions, with the exception of the above-mentioned *Volvox* ISG gene. Some extensins from carrot, tobacco and graminaceous species have an intron in their 3′ non-coding region. In graminaceous species, the intron sequence is very conserved when maize, teosinte and sorghum HRGP sequences are compared (Raz *et al.*, 1992). A similar intron has also been detected in the 3′ untranslated region of TPRP-F1 tomato PRP gene that will be described later. Introns in 3′ non-coding regions are very unusual in both animal and plant systems. They have been reported before, for instance, in the mouse major urinary protein genes. Another intron in a non-coding region has also been reported in the 5′ non-coding region of *Chlamydomonas reinhardtii* extensin. Other introns in a similar 5′ position have also been described in the *hsp*83 gene in *Drosophila melanogaster* and in the polyubiquitin gene in humans, chickens, sunflower, *Arabidopsis* and maize. Until now it has not been shown whether introns placed in extensin non-coding regions could have any regulatory function or are only trapped sequences.

The analysis of the proline repeats of HRGPs indicates the presence of the common motif SPPPP or $X(P)_n$ in dicots (group 0, Table 2). In many cases SPPPP is only a portion of a larger repeating sequence that can be assigned to two (Showalter & Rumeau, 1990) or three main subfamilies or groups (Table 2). The consensus sequence for group A repeats is SPPPPSPSPPPPYYYK. Variants for this sequence can be observed in Table 2. HRGP tomato repeats are mainly represented in this group. Group A repeats are also present in bean and petunia. Distant types of repeats would be the unrelated elements HAPP and PYHYP, present in tobacco HRGPnt3 and almond tree, respectively. SPPPPTPVYK is the main sequence for repeats belonging to class B, where the more distant element would be represented by the sequence SPPPPVH/A present in tomato Tom 5 and *Arabidopsis* extensins. Sometimes, but not always, these repeats alternate in the same extensin sequence with repeats belonging to group C, as can be seen when comparing groups B and C. Group B repeats are represented in tomato, *Arabidopsis*, oilseed rape, sugar beet, *Antirrhinum*, carrot, tobacco and petunia HRGP proteins and in Douglas fir. Group C repeats are mainly represented by the sequence SPPPPKK followed by a very degenerate tail. As can be observed in Table 2, repeats belonging to this group are present in tomato, melon, carrot, almond tree, tobacco, petunia and oilseed rape.

In the known HRGPs from monocotyledonous species, in gymnosperms and in algae the main repeated sequence is not SPPPP. In fact, this sequence is present only once in the maize HRGP sequence and it is completely absent in the rice HRGP, in *Antirrhinum* ptl 1, in Douglas fir dfPHRGP and class IV extensin from *Chlamydomonas reinhardtii*. The absence of SPPPP in Douglas fir dfPHRGP and *Chlamydomonas reinhardtii* Class IV extensins could be the result of a degenerative process, as their repeats can be included in groups A, B or C. This is not the case for graminaceous HRGP repeats that must be included in a new group D. Repeats rich in threonine (PPTYTP, PPTYKP, ATKPP and TPKPT) or without it SPKPP are observed in maize (Kieliszewski *et al.*, 1990; Stiefel *et al.*, 1988, 1990). In rice (Caelles *et al.*, 1992) the amino acid serine is almost completely absent, being replaced by glutamine residues, indicating that serine is not essential in itself for the HRGP function. In the case of maize, the sequence of the protein has been compared between different maize varieties to teosinte and sorghum (Raz *et al.*, 1992, see Table 2). It appears

that the protein is highly polymorphic, the number of repetitive elements being very variable in number but very conserved in the elementary sequence. It has been suggested that a recombinatory activity similar to that acting on satellite DNA may be responsible for this observation. In fact, the monocot sequences obtained so far all have in common a well-defined domain structure that includes a small region rich in glycine and tyrosine at the *N*-terminus, followed by a highly hydrophilic region, often containing histidine, and by the main repetitive sequence. A similar domain structure is also found in the tobacco $HRGP_{nt}3$ (Keller & Lamb, 1989) sequence expressed in the initiating lateral roots.

2. *Cellular localization of HRGP mRNA and polypeptides*

HRGPs have always been associated with cell walls on the grounds of their extraction properties and the correlation of their composition with the available data on the components of plant walls. However, how they interact with other wall components is at present not well understood. The possibility that these proteins form inter- or intra-molecular bonds through isodityrosine bridges has already been mentioned (Fry, 1986; Stafstrom & Staehelin, 1986*a*). As HRGPs are basic proteins containing in general lysine residues regularly spaced along the extended peptide backbone, an interaction in the wall between HRGP and the block polyanion regions of pectin seems possible. This interaction could be modulated by pH and $[Ca^{2+}]$ (Tierney & Varner, 1987). ϵ-amino groups of the lysine residues could also react with the reducing ends of polysaccharides. Extensins are also in general rich in histidine. As the histidine imidazol nitrogen has a pK of about 6 the charge on the nitrogen could vary as a consequence of physiological changes in the wall pH, and therefore the interaction with wall polyanions can be modulated. Enzymes acting on wall components have the ability to change these variables, allowing the wall to undergo the changes needed at different physiological states (Tierney & Varner, 1987; Showalter & Rumeau, 1990).

Several techniques such as western blot, tissue print immunoblots and immunoelectron or immunolight microscopy have been applied to the detection of HRGPs in distinct cell types. Different methodologies may give complementary and non-identical information. Immunolight microscopy of included sections detects proteins that are both extractable and attached to other cell wall components, whereas western blotting or tissue printing detects only the extractable fraction of the protein. In this sense, it is interesting to note the good correlation between the patterns of tissue printing obtained with immunological probes to detect extensin proteins or with DNA probes to detect their transcripts (Ye & Varner, 1991), indicating that pools of non-polymerized monomeric protein are mainly present in those cells active in HRGP gene transcription. A deduction from this fact is that the transcriptional control is essential for the expression of these genes, a fact that has been confirmed by promoter analysis, as will later be described.

Cassab *et al.* (1985) and Cassab & Varner (1987) developed an antibody against extensin purified from soybean seed coats. This antibody allowed the detection of the protein by western blotting in cell wall extracts from soybean seed coats at 16–18 d after anthesis. The amount of extensin increased during development, achieving the highest levels of detectable protein at 24 d after anthesis. Immunogold-silver staining and light microscopic immunocytochemistry studies in the seed coat allowed the detection of extensins mainly in the cell walls of both types of sclereid cells, the epidermal palisade cells and the hourglass cells, specially at the hilum region. The presence of HRGP associated with the parenchyma cells was much less obvious (Cassab & Varner, 1987). By immuno-tissue printing of soybean seeds, HRGPs were localized in the whole seed, being mainly associated with the seed coat, hilum and the vascular supply of the seed (Cassab & Varner, 1987). The association of HRGPs with sclereid cells involved in the ability of plant organs to withstand various strains, such as those resulting from stretching, bending, weight and pressure, without undue damage to the thin-walled softer cells such as parenchyma, may indicate that HRGPs help to confer these properties on sclereid cell walls (Cassab & Varner, 1987). In maize, an accumulation of HRGP mRNA is also observed in cells that will contribute to the resistance to mechanical stress (Stiefel *et al.*, 1990). Later, extensin was localized by immunoelectron microscopy in the cell wall of carrot roots, but it was absent from the expanded middle lamella using an antibody against purified glycosylated extensin-1 (Stafstrom & Staehelin, 1988). By immuno-tissue print and immunogold cytochemical approaches soluble extensin was localized in young soybean hypocotyls and roots using the same antibody against extensin purified from soybean seed coats (Ye & Varner, 1991). Soluble HRGPs were abundant in the hypocotyl apical region and in the root tip region, whereas in elongating and mature regions soluble extensin was present in some cortex cells around vascular bundles. In young soybean stems soluble HRGPs were found in the epidermal and cambial regions. After secondary growth soluble HRGPs appeared mainly in the cambial region; in young petiole they were found in epidermis and subepidermis cells. The association of high levels of HRGPs with vascular or provascular cells was shown not only by immunological techniques but also by *in situ* hybridization techniques, as will be discussed later (Ye & Varner, 1991; Stiefel *et al.*, 1990). A

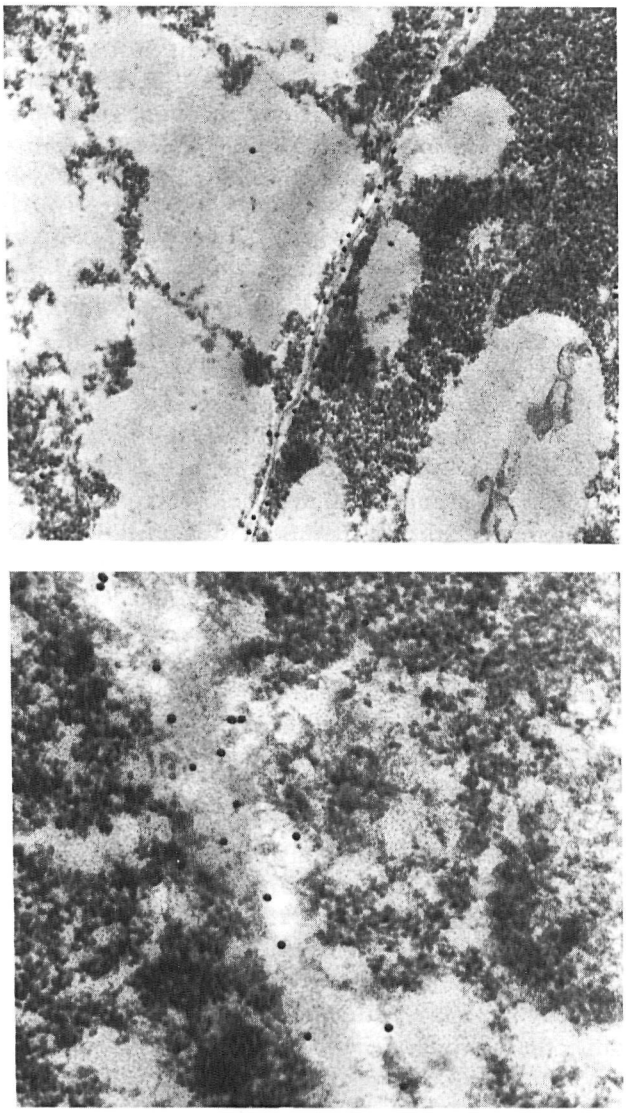

Figure 1. Immunocytochemical labelling of HRGP in cell walls from maize root tips observed by electron microscopy. Root-tip sections from 6-d-old maize seedlings were fixed in 4% paraformaldehyde, dehydrated in a graded ethanol series and embedded in Lowicryl K4M. Ultrathin sections were incubated with anti-maize HRGP serum and developed with protein A-gold complexes. Bars 0·1 μm (by courtesy of Dr M. D. Ludevid).

particular behaviour was reported for pine extensin-like protein (PELP) immunolocalized in secondary cells walls of early wood (Bao *et al.*, 1992). The different localization and function of this extensin-like protein is not the only difference when compared to extensins. This protein, although glycosylated, has a lower content of hydroxyproline and lysine than HRGPs, and there are also acid amino acids that may confer on the protein different possibilities for interacting with other wall proteins.

Antibodies raised against HRGPs purified from maize coleoptiles enabled extensin to be localized in the cell wall of maize root tips (Fig. 1) by immuno-electron microscopy (Ludevid *et al.*, 1990) as well as by light microscopy (Ruiz-Avila, Ludevid & Puigdomènech, 1991). Monoclonal antibodies raised against the PC-1 fraction from maize pericarp HRGP were used in western blot and tissue print analyses to localize the protein during maize kernel development (Hood, Hood & Fritz, 1991*b*; Fritz, Hood & Hood, 1991) and in the cell wall of maize tissues (Hood *et al.*, 1991*a*). Maize HRGPs extracted from stem node, plumule, mesocotyl, roots, embryos, pericarp and silk tissues had the same electrophoretic mobility, suggesting only one HRGP polypeptide (Hood *et al.*, 1991*b*; Ruiz-Avila *et al.*, 1991). Heterogeneity sometimes appeared when proteins were extracted from silk (maize style and stigma) cell walls (Hood *et al.*, 1991*a*) or when proteins, extracted at different times after pollination from pericarp cell walls, were compared (Hood *et al.*, 1991*b*). By tissue printing, maize HRGP was localized in the vascular bundle and the epidermis of stem, leaves and tassel stalks. Silk prints stained strongly and did not seem to show any cell-type specificity (Hood *et al.*, 1991*a*).

An antibody developed against a melon extensin subfraction, $HRGP_{2b}$, (Mazau *et al.*, 1988) enabled identification of the presence of HRGPs in roots from susceptible and resistant tomato cultivars infected by *Fusarium oxysporum* f. sp. *radicis-lycopersici* (Benhamou *et al.*, 1991*b*). This pathogen colonizes the vascular stele within a few days after root inoculation via intense invasion. However, in the genetically resistant tomato cultivars, fungal colonization is restricted to the outermost tissues, without ever reaching the endodermis. HRGP was shown to increase earlier and to a higher extent in resistant than in susceptible cultivars. HRGP in the compatible interaction seemed to appear as a result of wall damage, while in the incompatible interaction it was accumulated in the walls of uninvaded cells, thus indicating a possible role in the protection against fungal penetration. HRGPs were also observed in the papillae developed during the defence response in the intercellular spaces, suggesting that they may be involved in preparing them for their subsequent lignification (Benhamou *et al.*, 1991*b*). HRGPs were also accumulated in bean root nodules after infection by *Rhizobium leguminosarum* bv. *phaseoli* (Benhamou *et al.*, 1991*a*). HRGPs accumulated mainly in walls of infected cells and in peribacteroid membranes surrounding groups of bacteroids as well as in their internal ramifications (Benhamou *et al.*, 1991*a*). Recently, using an antibody against maize HRGP it was shown that in the interface between the plant and the fungus in maize mycorrhizae the location of HRGP accumulation was in accordance with the unspecific type of response to fungal invasion of plant tissues (Bonfante, P., personal communication).

The use of the tissue printing technique on nitrocellulose paper allowed rapid location of HRGP mRNAs in different plant tissues from a number of plant species (Ye & Varner, 1991). mRNA tissue

printing using a carrot genomic probe (pDC5A1) led to detection of HRGP mRNAs in young hypocotyls, stems and petiole and seed coats from soybean. In young soybean stems HRGPs were expressed most heavily in cambium cells, and in the epidermis region. In older soybean stems, extensins were exclusively expressed in cambium cells. A similar vascular pattern of expression was observed in soybean petioles and seedcoats (Ye & Varner, 1991). The same study was made in different tissues of Solanaceae species such as tomato petioles and stems, tobacco and petunia leaves and stems. In all cases, although with some different preferences, HRGP mRNAs were associated with provascular cells (Ye *et al.*, 1991). The same effect was reported in maize, where the accumulation of HRGP mRNA was observed for instance in provascular cells (Fig. 2), in developing coleoptiles and in the procambium of germinating embryo. This expression was transient and, as in older vascular cells, the HRGP mRNA accumulation disappeared (Stiefel *et al.*, 1990; Ruiz-Avila *et al.*, 1992).

3. *Expression of HRGP genes*

The data using histological detection techniques indicate that the accumulation of HRGP mRNA depends on tissue-specific factors, as well as on the physiological state of the plant. In general, HRGP mRNA accumulates in meristematic plant tissues, and in particular a high accumulation has been observed in radicular meristems. This is specially evident in tobacco (Memelink *et al.*, 1987), where an increased accumulation of HRGP mRNA was observed when cytokinin synthesis genes were introduced into transgenic plants. Histochemical data also show specific accumulation in vascular tissues and in response to external trauma to the plant. This latter effect has been specially analyzed in dicotyledonous species. A high-expression specificity is also observed for extensin and extensin-like genes, expressed only in mature transmitting style tissues (Baldwin *et al.*, 1992; Chen *et al.*, 1992; de S. Goldman *et al.*, 1992). If the proteins were located in the wall, their function could be related to the flexibility of the style wall needed during pollen grain germination in order to help cell–cell interactions rather than having a defensive role, as they do not seem to be related to the wounding or stress responses.

In monocots, HRGP mRNA accumulation has been studied mainly in maize. Maize HRGP protein is accumulated in the pericarp (Hood *et al.*, 1988, 1991*b*) and in cell suspension cultures (Kieliszewski & Lamport, 1987; Kieliszewski *et al.*, 1990). In general, maize HRGP mRNA has been shown to accumulate in tissues rich in dividing cells and in response to wounding (Ludevid *et al.*, 1990) and ethylene (Tagu *et al.*, 1992). However, by *in situ* hybridization the presence of an increased level of HRGP mRNA was observed in provascular cells (Stiefel *et al.*, 1990). A tissue-specific control has also been observed in embryonic tissues where a low (or nil) level of expression was observed in the scutellum at both protein and mRNA level (Ruiz-Avila *et al.*, 1991).

As indicated above, plant defence mechanisms

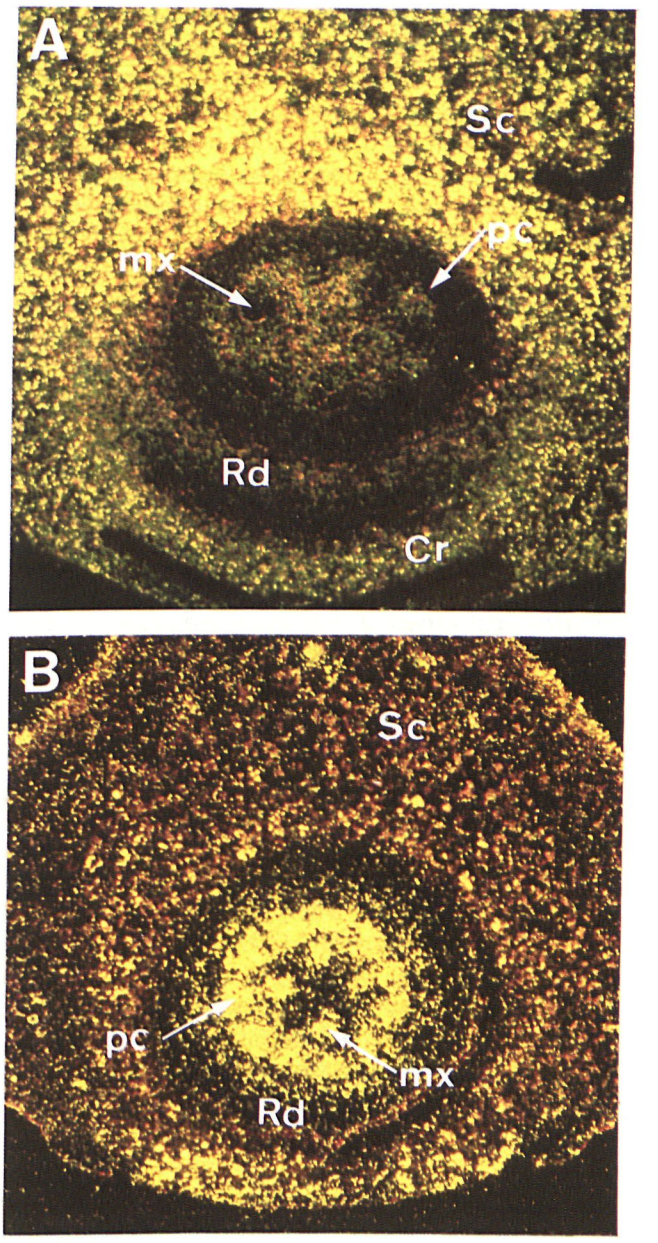

Figure 2. Comparison of the pattern of *HyPRP* and *HRGP* mRNA, accumulation in immature maize embryos by *in situ* hybridization. A. *In situ* hybridization of an *HyPRP* antisense RNA probe with a radicle transverse section from an embryo 18 DAP. B. *In situ* hybridization of an *HRGP* antisense RNA probe with a radicle transverse section from a decussate maize mutant embryo 15 DAP (by courtesy of Drs M. Josè and Dr L. Ruiz). Bar 100 μm. The sections were examined under a dark-field microscope with a green or yellow filter, respectively. Sc, Scutellum; mx, metaxylem precursor cells; pc, procambium; Rd, radicle; Cr, coleorrhiza.

include HRGP accumulation. Defence mechanisms are developed by plants to tolerate wounding or the action of pathogenic agents, and they include deposition of lignin-like material, synthesis of hydrolytic enzymes such as β-glucanases and chitinases, gene activation of the phenylpropanoid metabolic enzymes (PAL, CHS or 4CL) and phytoalexin synthesis (Cramer *et al.*, 1985). Along with these reactions, different HRGP transcripts are also induced or repressed in a special manner. In fact one of the roles proposed for HRGP proteins in the cell wall has been the control of cell expansion and resistance to invading pathogens, by virtue of its cross-linking ability, which could render the wall indigestible by invading pathogens. Extensins are resistant to proteases, and could only be hydrolyzed by trypsin if the arabinose residues were removed (Lamport, 1977; Lamport & Epstein, 1983). In a different manner extensins could also act as polycations, agglutinating bacteria and thereby preventing their spread (Leach *et al.*, 1982).

The response of HRGP to wounding at mRNA level was first detected using DC5A1 genomic extensin probe against carrot storage root RNA. Twenty-four h after wounding, two transcripts of different size (1·5 and 1·8 Knt) were recognized by the probe (Chen & Varner, 1985*b*). Both transcripts come from the same gene but from different transcription initiation sites placed at a distance of 300 bp between themselves. Transcription from one or other initiation site could be under different regulatory signals. In wounded carrot roots, the accumulation of the 1·5 Knt transcripts began to occur after 8 h, the maximum level being attained after 24 h (Tierney, Wiechert & Pluymers, 1988). A more rapid response was detected by Ecker & Davis (1987) in peeled stored carrot roots after 1 h of incubation in a stream of moist air. Under these conditions the 1·5 Knt transcript showed a dramatic increase in contrast to the 1·8 Knt one. Wounding as the result of the process of protoplast preparation from carrot cells resulted also in the induction of the 1·5 Knt transcript after 16 h with a maximum level also after 24 h (Ecker & Davis, 1987).

The response of bean HRGP genes Hyp 2.13, Hyp 3.6 and Hyp 4.1 to excision-wounding in hypocotyls was studied by Corbin *et al.* (1987). Within 1·5 h, Hyp 3·6 mRNA was strongly induced at almost its maximum level while Hyp 2·13 and Hyp 4·1 mRNAs were induced later and reached a maximum only after 12 h. Hyp 3·6 mRNA levels decayed between 12 and 24 h, whereas the other mRNAs remained at maximum levels. Nuclear run-off transcription assays demonstrated that the wound induction of extensin mRNA accumulation in bean hypocotyls was a result of transcriptional activation (Lawton & Lamb, 1987). Class I and Class II tomato extensins were expressed preferentially in basal and apical tomato stems respectively (Showalter, Butt & Kim, 1992) and in roots but not in leaves. After wounding they decreased in roots, but in stems they were locally but not systematically accumulated after 8–12 h. Thereafter Class II tomato extensin mRNA levels decreased. Sunflower HaGX 3 gene responded to wounding in leaves but not to ethylene (Adams *et al.*, 1992).

After wounding or pathogen infection, ethylene is synthesized by plants. Ethylene treatment of plants is able to mimic the defence mechanisms and activates phenylpropanoid metabolic enzymes. Ecker & Davis (1987) showed that, in rapidly growing carrot roots, 4-CL and CHS enzyme activity increased 20 and 50 times after 6 and 24 h respectively of ethylene treatment. Instead, PAL enzyme activity was still increasing after 48 h (Ecker & Davis, 1987). Under the same conditions, using the genomic carrot extensin probe pDC5A1, the expression of the 1·5 and 1·8 Knt transcripts was only slightly affected. Instead, when cold-stored carrot roots were treated with ethylene and oxygen for 72 h, the 1·8 Knt transcript was clearly induced while the 1·5 Knt transcript decreased. This ethylene response was not inhibited if roots had been wounded previously (Tierney *et al.*, 1988). This behaviour of carrot extensin transcripts after ethylene treatment is just the opposite to the one described above after wounding, when the 1·5 Knt transcript instead of the 1·8 Knt one was actively induced (Ecker & Davis, 1987). As mentioned above, both transcripts come from the same gene (Chen & Varner, 1985*b*), but they respond differently to different attacks (Ecker & Davis, 1987), showing that they are under the control of different regulatory signals (Ecker & Davis, 1987). Class I and Class II tomato extensin RNA were both accumulated by enclosure and by ethylene treatment (Showalter *et al.*, 1992).

Since the plant cell wall represents the boundary interface with pathogens one may expect that, after infection, active defence reactions, such as HRGP accumulation, occur at the cell surface level. Infection of bean (*Phaseolus vulgaris* L.) hypocotyls with *Colletotrichum lindemuthianum*, the causal agent of anthracnose, allowed detection of HRGP transcripts using the extensin tomato genomic probe Tom 5. In an incompatible interaction, such as the one involving a resistant host, there was an early increase in HRGP mRNA correlated with expression of hypersensitive resistance, whereas in a compatible interaction, marked accumulation of HRGP mRNA occurred as a delayed response at the onset of lesion formation. In both interactions, mRNA accumulation was observed in uninfected cells distant from the site of fungal inoculation, indicating intercellular transmission of an elicitation signal (Showalter *et al.*, 1985). Later, Corbin *et al.* (1987) using bean HRGP probes, Hyp 3·6, Hyp 2·13 and Hyp 4·1, observed that the three respective transcripts were induced by both the compatible and the incompatible strains of

the fungus. Hyp 2·13 was preferentially induced in the compatible reaction, whereas Hyp 3·6 and Hyp 4·1 were strongly induced in both types of interaction. These results were confirmed by *in situ* hybridization using the Hyp 4·1 probe. In the incompatible interaction HRGP transcripts were induced in the epidermal and cortical cells directly below the inoculation site and in the perivascular parenchymal tissue of uninfected tissues. In the compatible interaction HRGP transcripts were accumulated only in the perivascular parenchymal tissue (Templeton *et al.*, 1990). The carrot extensin probe also enabled identification of the induction of HRGP transcripts in sunflower stem base plants 2 or 3 d after infection by *Sclerotinia sclerotiorum* (Lib.) de Bary (the causal agent of white mould) in a tolerant or susceptible sunflower line. Oxalic acid, the toxin produced by the fungus, was shown to be able to induce HRGP transcripts, thus behaving as an elicitor (Mouly, Rumeau & Esquerré-Tugayé, 1992).

Elicitors by themselves are able to induce similar defence mechanisms to those induced after pathogen infection. Showalter *et al.* (1985) using the tomato extensin probe, Tom 5, observed that bean cells treated only with elicitors accumulate extensin mRNAs. After treatment with *C. lindemuthianum* elicitors, HRGP transcripts appeared after 4 h and increased between 6 and 12 h, remaining stabilized afterwards. The response was slow and maintained, in contrast to phytoalexin synthesis which was fast and transient. When the same study was made using probes from the homologous system (Corbin *et al.*, 1987), the Hyp 2·13, Hyp 3·6 and Hyp 4·1 transcripts were recognized after 24 h. In bean, a similar defence response was produced when the reduced form of glutathione (GHS) was supplied to suspensions of bean cultured cells (Wingate, Lawton & Lamb, 1988).

A crude endogenous carrot cell-wall fragment fraction also induced HRGP transcripts in carrot cell suspension cultures after 5 d of treatment. This behaviour suggested that cell damage may release cell wall factors that by themselves can regulate the expression of defence-related genes and work as endogenous elicitors (Tierney *et al.*, 1988).

Other conditions have been described to affect the level of HRGP mRNA. A threefold increase in HRGP mRNA has been described in epicotyls after germination of pea seedlings acclimatized to cold conditions. Extensin increase was supposed to help by conferring a major resistance to collapse caused by freeze-induced dehydration (Weiser, Wallner & Waddell, 1990). HRGPs might also behave as heat-shock proteins, increasing in response to heat treatment (Stermer & Hammerschmidt, 1987; Showalter & Varner, 1989). Red-light treatment of etiolated pea epicotyls increases the level of wall-bound hydroxyproline, and the effect can be reversed by far-red light, suggesting a role of phytochrome in HRGP regulation (Pike, Lystash & Showalter, 1979). Finally, an accumulation of hydroxyproline in cell walls has also been suggested to be induced by gravity in morning glory stems (Prasad & Cline, 1987).

The results presented above indicate that, in the dicot plants studied, each one of the genes coding for HRGP is induced by wounding or ethylene in a specific way, being under the control of different stress-signal systems. In monocotyledonous species the situation may be different, because the HRGP gene seems to be a much simpler system. The data obtained so far are consistent with a single or a very low number of genes coding for HRGP in maize (Stiefel *et al.*, 1990), sorghum (Raz *et al.*, 1991) and rice (Caelles *et al.*, 1992). In any case it has been shown that the HRGP probes from these cereals detect, in inbred lines only, a transcript that is developmentally regulated as well as induced by wounding (Ludevid *et al.*, 1990) and ethylene (Tagu *et al.*, 1992). The induction appears to be dependent on the organ and the age of the plant. The two responses may be separated in relation to a typical marker of cell division, histone H4 mRNA, while in normal plant development the two mRNAs are accumulated in a parallel way. Histone H4 mRNA accumulation is not increased by ethylene, while HRGP mRNA levels increase, in a tissue-specific way, several-fold (Tagu *et al.*, 1992). It seems that, while in dicots the different types of induction are carried out by specific genes, in cereals a single gene is able to respond, through distinct control mechanisms, to the different physiological situations.

4. *HRGPs' regulatory sequences and proteins*

HRGP genes provide a useful model in plants to examine regulatory mechanisms associated with events such as pathogen infection, wounding and development. Elucidation of the promoter regulatory elements and of proteins responsible for HRGP gene regulation can now be studied. Currently only a few studies have been made using the carrot HRGP gene pDC5A1 promoter. Granell *et al.* (1992) studied the response of this gene upon wounding by electroporation of protoplasts from carrot cellular suspensions with the pDC5A1 promoter fused to GUS as a reporter. The first 719 bp of the 1·5 Knt transcript promoter activated by wounding proved to be enough to give maximum expression. Two regulatory elements were identified in this region, the first one located between −719 and −658 and the second one between the TATA boxes of the 1·8 and 1·5 Knt transcripts. The first regulatory element is recognized by an extensin-binding protein (EBP) present only in nuclear extracts prepared from carrot protoplasts. Two other factors, b_1 and b_2, present in cell suspensions but not in protoplast extracts, appear to bind to a non-relevant promoter region

between −609 and −474. The same region (−594 to −554) had been reported before (Holdsworth & Laties, 1989*a*, *b*) to bind factors EGBF-1 and EGBF-2 extracted only from adult carrot roots before induction of gene expression by wounding or ethylene. As these factors are not found in any other vegetative tissue they are considered to be root-specific repressors of extensin gene expression. EGBF-1 binding activity is present in phloem extracts, while EGBF-2 is present in xylem extracts. Their mobilities are slightly different, but they recognize the same AT-rich promoter region. In extracts from wounded roots the absence of EGBF-1 binding is the result of an inhibitory activity present in these extracts (Holdsworth & Laties, 1989*b*). This activity is heat sensitive. This inhibitory activity is absent in extracts from roots treated with ethylene, indicating that EGBF-1 may be controlled through different factors by ethylene and wounding.

Several studies on other extensin genes have been carried out. The 1·3 Kb promoter sequence from the 5′ flanking region and first 27 nucleotides of the $HRGP_{nt}3$ tobacco HRGP gene promoter are sufficient to allow specific expression of the promoter fused to a *GUS* reporter gene in transformed plants. Expression is associated with those cells initiating secondary root growth at the level of the pericycle and endodermis (Keller & Lamb, 1989). A kilobase from the oilseed rape (*Brassica napus*) ExtA gene promoter fused to GUS allows expression in root phloem of transformed tobacco plants whilst, when plants are transformed with the promoter and coding regions of the ExtA gene, mRNA transcripts with the correct size are localized in roots. However, a basal level of expression is also observed in the hypocotyl, probably as the result of using a heterologous system for the expression (Shirsat *et al.*, 1991). On the other hand, regulatory signals of protein deposition have been studied by transforming tobacco protoplasts with the tobacco npExt gene (de Loose *et al.*, 1991). When it was found that only the first 18 amino acids of the signal peptide are necessary to secrete the protein from the protoplast, showing that vacuolar processing signals are absent.

In monocots, the promoter sequences of the maize HRGP gene have been studied. On the one hand, a region of around 500 bp has been shown to be highly conserved when genomic sequences from different maize varieties, teosinte, and sorghum were compared (Raz *et al.*, 1992). Interestingly, this region shows a number of well-conserved boxes, including one that is identical to a box shown to be responsible for ethylene induction in a parsley chitinase gene (Broglie *et al.*, 1989). This region also contains the main hypersensitive site to nuclease digestion (Vallés *et al.*, 1991). Functional analysis of this region has been carried out by microbombarding (Tagu *et al.*, 1992) and it was shown that 1 kb construction of the maize HRGP promoter seems to keep some of the qualitative features observed in the gene (Fig. 3).

III. PROLINE-RICH PROTEINS (PRPS)

1. *Features of PRPs*

Although extensins are the best-studied group of plant proteins containing repetitive sequences containing a high proportion of proline, other types of proline-rich proteins have also been identified that differ from extensins both in the sequence of the repetitive elements [for instance, they lack the characteristic Ser-$(Hyp)_4$ motif] and in the features of the expression of their genes. These proteins were mainly identified through recombinant DNA studies and, according to their sequences and their expression properties, they can be classified into a number of different groups. A summary of these sequences can be found in Table 3; they are in general named PRPs. The first PRP sequence was described in wounded carrot root cDNA (Chen & Varner, 1985*a*), whilst the ones most often called PRPs were identified in soybean. Different cDNAs were isolated from germinated soybean hypocotyls (Averyhart-Fullard, Datta & Marcus, 1988; Datta, Schmidt & Marcus, 1989), from soybean cell cultures grown in the pressure of auxin (Hong, Nagao & Key, 1987) and from soybean seed coats (Lindstrom & Vodkin, 1991). In this group the sequence of a soybean gene (ENOD 2) induced by *Rhizobium* during the formation of root nodules can also be included (Franssen *et al.*, 1987). In monocots, a PRP has also been described in wheat and called WPRP 1 (Raines *et al.*, 1991).

Three genomic clones corresponding to PRPs were also isolated from soybean. The first one is soybean SbPRP1 (Hong *et al.*, 1987) identical to two previously described cDNA clones [pTU04 described in Hong *et al.* (1987) and pB 1-3 described in Lindstrom & Vodkin (1991) although the last one has a duplication that adds five extra repeating units to the coding region]. The second one is soybean RPRP 3 (Datta & Marcus, 1990), identical to soybean SbPRP 2 (Hong, Nagao & Key, 1990) and to the cDNA sequence described by Datta *et al.* (1989). The third soybean genomic sequence is SbPRP3 (Hong *et al.*, 1990).

PRP sequences show a large heterogeneity in their respective amino acid compositions. The deduced proteins from soybean SbPRP 1, SbPRP 2 and SbPRP 3 sequences lack His and Ser, have moderate amounts of acid amino acids such as Glu and high levels of Tyr and Lys. Wheat WPRP1 lacks His, Ser and Tyr and has a particularly high content in basic and acid amino acids, in particular Lys and Glu. ENOD2 contains His, Tyr and Lys in similar amounts and a high level of Glu but lacks Ser. Carrot p33 contains high levels of His and Lys, and Ser, Tyr and Glu in moderate amounts.

Table 3. *cDNA and genomic clones encoding proline-rich proteins*

Name	Nature	Plant	aa	Reference
PRPs				
p33	Partial cDNA	Wounded carrot roots	211	Chen & Varner (1985*a*)
pTU04	Partial cDNA	Soybean cell culture growth in the presence of auxin	nd	Hong *et al.* (1987)
pB 1-3	Complete cDNA	Soybean seed coat	nd	Lindstrom & Vodkin (1991)
SbPRP 1	Genomic	Soybean	**256**	Hong *et al.* (1987)
1A10	Partial cDNA	Soybean cell culture	120	Averyhart-Fullard *et al.* (1988)
1A10-2	Complete cDNA	Soybean axis	**230**	Datta *et al.* (1989)
RPRP 3/SbPRP 2	Genomic (1A10-2)	Soybean	**230**	Datta & Marcus (1990) Hong *et al.* (1990)
SbPRP 3	Genomic	Soybean	**90**	Hong *et al.* (1990)
ENOD 2	Partial cDNA	Soybean root nodules	241	Franssen *et al.* (1987)
WPRP 1	Complete cDNA	Wheat	**378**	Raines *et al.* (1991)
Hybrid PRPs with Cys				
PvPRP 1	Complete cDNA	Elicitor-treated bean cells	**297**	Sheng *et al.* (1991)
TPRP-F 1	partial cDNA	1-wk-old tomato fruit	313	Salts *et al.* (1991)
TPRP-F 1	Genomic	Tomato	**346**	Salts *et al.* (1992)
DC 2.15	Complete cDNA	Carrot cell culture growth in 2,4-D free medium	**137**	Aleith & Richter (1990)
zmHyPRP	Genomic	Maize	**301**	Josè-Estanyol *et al.* (1992)
Other PRPs				
SF 18	Partial cDNA	Sunflower flower	nd	Herdenberger *et al.* (1990)
SF 19	Partial cDNA	Sunflower flower	nd	Herdenberger *et al.* (1990)

aa, Number of protein aminoacids including the signal peptide; complete sequences are indicated in bold figures. nd, not determined.

PRP proteins were purified from different soybean tissues. The protein corresponding to the SbPRP 2 genomic sequence was isolated from soybean cell cultures (Averyhart-Fullard *et al.*, 1988; Datta *et al.*, 1989). This protein was separated in two fractions: RPRP 2 (28 kDa) and RPRP 3 (33 kDa), both having similar amino acid composition. Their close correspondence to the cDNA 1A10-2 and genomic SbPRP 2 sequences is based on the amino acid content of the major chymotryptic peptide and on the sequence of the first 49 amino acid residues. RPRP 3, which has only half of its proline residues hydroxylated and is not glycosylated, is more stable than deglycosylated extensins and bovine serum albumin to moderate alkaline treatment, and refractory to staphylococcal protease (Drapeau, 1977) and proteinase K (Ebeling *et al.*, 1974). This behaviour was explained as the result of a novel three-dimensional structure of the protein as, theoretically, it appeared to be sensitive to these enzymes. These are important differences from dicot extensins, which have nearly all their proline residues hydroxylated, are exhaustively glycosylated and can be hydrolyzed by proteases when deglycosylated and also by alkali. The same protein was also purified from the apical hook of etiolated 4-d-old germinated soybean seedlings (Kleis-San Francisco & Tierney, 1990) as determined by sequencing the first 18 amino acid residues. Finally, the protein corresponding to the genomic sequence SbPRP 1 was isolated from the soybean seed coat (Lindstrom & Vodkin, 1991) as determined by sequencing the first 14 amino acids.

Other PRPs have recently been identified, and all of them can now be classified in relation to their sequences (Table 3). PRPs have in common a signal peptide that might be used for the transport of the protein out of the cell. When the distribution of their proline repeats is analyzed with respect to the whole protein they can be arranged in three groups.

(1) PRPs with proline repeats along all the protein and without Cys. They have been described above.

(2) Hybrid PRPs (HyPRP). These PRPs contain a first domain with proline repeats and a second one which is hydrophobic and rich in cysteine and without proline repeats. From this group two classes of cDNA sequences were isolated that detect mRNA with a different pattern of accumulation. The first one corresponds to the full-length cDNA PvPRP 1 from bean-cell suspension cultures treated with fungal elicitors. It is expressed everywhere in bean plants and it is rapidly down-regulated by fungal elicitors and wounding (Sheng, d'Ovidio & Mehdy, 1991). The second one, TPRP-F 1, was isolated from a young tomato fruit cDNA library (Salts *et al.*, 1991) and the corresponding genomic sequence is also available (Salts *et al.*, 1992). The tissue-specific expression of this gene in tomato and the sequence of its hydrophobic domain are similar – although having different proline-rich repeats – to a genomic sequence isolated from a maize genomic library which codes for an mzHyPRP (Josè-Estanyol, Ruiz-Avila & Puigdomènech, 1992). It is interesting to note the homology of tomato TPRP-F 1 and maize HyPRP hydrophobic domains with the sequence of

Table 4. *Amino acid sequence repeats in PRPs*

Group	Repeat	Number of repeats (name, plant)
A	PPVYK	29 (SbPRP 1, soybean); 19 (1A10, soybean); 17 (SbPRP 2, soybean); 6 (SbPRP 3, soybean); 1 (p33, carrot)
	PPYV	16 (zmHyPRP, maize)
	PPVYT	3 (P33, carrot); 1 (SbPRP 1, soybean)
	PPVKK	1 (SbPRP 1, soybean)
	PPYKK	2 (SbPRP 3, soybean)
	PPVHK	5 (p33, carrot)
	PPVEK	3 (1A10, soybean); 6 (SbPRP 1, soybean); 16 (SbPRP 2, soybean)
	PPVEN	1 (SbPRP 2, soybean)
	PPVED	1 (SbPRP 3, soybean)
	PPTEK	1 (SbPRP 2, soybean)
	PPHEK	17 (ENOD 2, soybean)
	PPYGK	1 (SbPRP 2, soybean); 1 (SbPRP 3, soybean)
	PPIEK	2 (SbPRP 1, soybean); 1 (1A10, soybean)
	PPIHK	3 (p33, carrot)
	PPIYK	1 (1A10, soybean); 4 (SbPRP 1 soybean); 2 (SbPRP 2, soybean)
	PPI/HVK/S	8 (TPRP-F 1, tomato)
	PPXTPK/T	8 (TPRP-F 1, tomato)
	PPPEYQ	6 (ENOD 2, soybean)
	PPPEHQ	3 (ENOD 2, soybean)
	PPEHQ	2 (ENOD 2, soybean)
B	PEPK	43 (WPRP 1, wheat)
	MPKPEPKPEPKPEP	14 (WPRP 1, wheat)
	PEPMPK	16 (WPRP 1, wheat)
	PMPK	4 (WPRP 1, wheat)
	PX	10 (DC 2.15, carrot)
C	PVHPPLNPP	1 (PvPRP 1, bean)
	PPHPPLKPPV	1 (PvPRP 1, bean)
	PIHPPLNPPV	1 (PvPRP 1, bean)
	PVHPPVKPPV	3 (PvPRP 1, bean)
	PVHPPV	1 (PvPRP 1, bean)
	PVHP	1 (PvPRP 1, bean)
	PV/LPPL/IP	nd (SF 19, sunflower)
D	PPTPRPS	7 (zmHyPRP, maize)

D, Aspartic acid; E, glutamic acid; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; S, serine; T, threonine, V, valine; X, any amino acid; Y, tyrosine; nd, not determined. Amino acids that are equally frequent in a position are linked by a slash (/). References for cDNA and genomic clones are given in Table 3. PX means the successive alternation of PXPXPX... up to 10 times.

a carrot cDNA DAUCA DC 2.15 expressed in early stages of carrot somatic embryos, which contains a small proline-rich domain formed by the sequence Pro-X, where X is frequently Thr (Aleith & Richter, 1990) and with a short hydrophobic seed protein from soybean (Odani *et al.*, 1987). This protein is related to a large family of defence, storage, and probably transport proteins (Henrissat, Popineau & Kader, 1988). In particular, the structure of these HyPRPs is similar to a family of storage proteins (Kreis & Shewry, 1989) formed by repetitive proline-rich and hydrophobic domains. In maize a group of storage proteins, the γ-zeins, share these features (Prat, Pérez-Grau & Puigdomènech, 1987). The amino acid composition of hybrid PRPs is not identified in the different proteins studied, independently of which domain we study. Another characteristic of the maize hybrid PRPs is that between the N-terminal signal peptide and the proline-rich domain there is a short domain without proline repeats. Ser, Gly and His are the most usual amino acids for mzHyPRP, and His for PvPRP1 in this region.

(3) Other PRPs. Here we include SF 18 and SF 19 partial cDNA clones (Herdenberger *et al.*, 1990; Evrard *et al.*, 1991) specific from sunflower anther epidermis with different proline repeats.

Different repeats are characteristic of the proline-rich sequences just described (Table 4). In soybean, PPVYK is the most represented repeat, with changes such as Y, I and H for V, or K, E, G and H for Y, or D, N and T for K. A similar repeat is observed in maize HyPRP (PPYV). PPVYK and their variants are not exclusive to PRPs, as they are also present in HRGPs in addition to the SPPPP motif in proteins containing extensin group B repeats (see Table 2). Wheat WPRP 1 and bean PvPRP 1 have a second and third class of repeats (PEPK) and (PVHPPVKPPV) respectively with their derivates. These repeats are not related to the general element PPVYK just

described. The maize HyPRP PPTPRPS repeat contains Thr and Ser as in the maize HRGP PPTYPSPKPP repeat, but in a different order. The presence of Ser and Thr, although normal in extensin repeats, is unusual in the PRP ones, as can be seen by comparing Tables 2 and 4. We can also observe that while bean, sunflower and wheat PRP repeats are distant from the PPVYK family, maize HyPRP repeats also appear divergent, but more close to the PRP general motif PPVYK (Table 4) and to maize HRGP repeats (Table 2).

2. *PRP immunolocalization*

The presence of PRPs in the cell wall has currently only been determined for soybean SbPRP 1 and SbPRP 2, as they have been purified using the methods characteristic for the isolation of cell wall proteins. For the other PRPs the cell wall location is only speculative and yet to be determined. Cell wall PRPs were immunolocalized in several dicot species using an antibody raised against soybean RPRP 3, which reacts with the three main soybean PRPs (Marcus, Greenberg & Averyhart-Fullard, 1991; Ye *et al.*, 1991). In young soybean roots and hypocotyls, PRPs were associated with protoxylem cell walls and the corner walls of the cortex. In older plants, PRPs were localized in the xylem vessel elements of the young stem and in phloem fibres in the older stem. Similar results were observed when tomato, petunia, potato and tobacco stems were studied. These results seem to indicate that PRP localization is related to the pattern of lignification (Ye *et al.*, 1991).

Antibodies against SbPRP 2 allowed the study of the rapid insolubilization (i.e. 2 min) of soluble pre-existing SbPRP 2 protein after fungal elicitor or glutathione treatment of bean or soybean cells, as well as after wounding of etiolated bean hypocotyls and in tissues subject to mechanical stress (Bradley, Kjellbom & Lamb, 1992). Insolubilization involves H_2O_2-mediated oxidative cross-linking, probably through Tyr, and in some experiments low levels of dimeric and tetrameric forms of protein were observed. As has been proposed, the stimulus-dependent cross-linking provides a mechanism for rapid hardening of the wall as a protection against environmental stresses (Bradley *et al.*, 1992). This theory might also be extended to HRGP proteins and other cell wall proteins containing tyrosine.

3. *Expression of PRP genes*

The accumulation of mRNA coding for PRPs shows specific patterns of distribution during development. These patterns seem to be specific for the different classes of PRP. For instance, wheat WPRP 1 only shows some preferential expression in meristematic tissues, and no response upon wounding is observed in the leaves (Raines *et al.*, 1991). In contrast, each soybean PRP is developmentally regulated in a characteristic way. SbPRP 1 is mainly expressed in mature roots and hypocotyls of germinating seedlings (Hong, Nagao & Key, 1989), being induced by water deficit in elongating cells from hypocotyl (Creelman & Mullet, 1991). SbPRP 2 is expressed in apical and elongating hypocotyls and in elongating and maturing roots. SbPRP 3 has limited expression in mature and elongating hypocotyls, but it is mainly expressed in 3-wk-old stems and leaves. All of the PRP genes are expressed in the soybean seed pod, especially SbPRP 3, and in the seed coat, although SbPRP 2 does not appear until days 24 and 28 after anthesis. No mRNA corresponding to these probes is detected in the cotyledons, but all of them are present in soybean cultured cells (Datta *et al.*, 1989; Hong *et al.*, 1989). A particular behaviour was established for SbPRP 1, in particular a correlation between the expression of the anthocyanin-related I gene and the quantitative levels of SbPRP 1 (Lindstrom & Vodkin, 1991). SbPRP 1 production was stimulated in yellow Richland soybean seed coats by the dominant genotype I/I. This genotype prevents the accumulation of anthocyanin pigments in the vacuoles of the seed-coat palisade cells. The same correlation was not found for SbPRP 2, which is synthesized later in the seed coat development and is not affected by the anthocyanin-related I gene (Lindstrom & Vodkin, 1991).

Cells responsible for PRP expression in soybean were analyzed in more detail by *in situ* hybridization (Wyatt, Nagao & Key, 1992). SbPRP 1 mRNA was expressed in phloem and xylem cells of soybean hypocotyls, and moreover in epidermal cells in the elongating and mature regions of the hypocotyl, as well as in lignified cells surrounding the hilum of mature seeds. SbPRP 2 mRNA was present in cortical cells and in the vascular tissue of the hypocotyl, especially cells of the phloem and in the inner integuments of the mature seed coat. SbPRP 3 mRNA was localized specifically in the endodermoid layer of cells surrounding the stele in the elongating region of the hypocotyl, as well as in the adaxial epidermal cells of leaves and in the upper epidermis of germinating cotyledons.

Carrot p33 is expressed in wounded carrot roots (Chen & Varner, 1985*a*) and it responds faster to wounding than extensin in this organ. Its expression is detected after 1 h, its maximum after 2 h, and this is maintained for 24 h, while extensin does not begin to be expressed until 8–12 h after wounding (Tierney *et al.*, 1988). This is not the case in graminaceous species, where maize HRGP extensin is detected 15 min after mesocotyl wounding (Tagu *et al.*, 1992). Carrot p33 does not seem to respond to ethylene; moreover ethylene is able to inhibit carrot p33 response to wounding or to repress it. In the same manner, when carrot suspension-cell cultures are

incubated in the presence of a crude elicitor fraction for 2 or 5 d, no accumulation of p33 mRNA can be observed, while extensin is induced after 5 d (Tierney *et al.*, 1988). It is interesting to note that until now a positive response of PRP genes to wounding has only been reported for carrot p33, and that no positive response of a PRP gene to ethylene, elicitors or fungal attack has been reported. Instead, bean hybrid PvPRP 1 decreases dramatically after bean suspension cells have been treated with elicitors and after hypocotyl wounding (Sheng *et al.*, 1991). In the same way ENOD 2 gene expression is associated with nodule morphogenesis and not with the infection process (Franssen *et al.*, 1987).

Hybrid PRPs from tomato TPRP-F 1 (Salts *et al.*, 1991, 1992) and maize HyPRP (Josè-Estanyol *et al.*, 1992) have a highly specific pattern of expression, being only expressed in young tomato fruit and immature maize embryos respectively. *In situ* hybridization studies on maize embryo sections (Josè-Estanyol *et al.*, 1992) indicated that HyPRP is expressed in scutellum cells and in non-vascular cells from the immature embryo axis (Fig. 2). Northern blotting studies confirm these results and also indicate a basal level of expression of HyPRP in the ovary just prior to pollination. Maize HyPRP could be negatively regulated by abscisic acid (ABA), and the expression of the gene is retarded until later embryogenesis in viviparous 2 (vp2) maize mutants defective in ABA. Interestingly, an ABA-responsive element (Guiltinan, Marcotte & Quatrano, 1990) is present in its promoter.

SF18 and SF19 genes from sunflower also have a high specific cellular and developmental expression. *In situ* hybridization studies have allowed the location of their expression in a single-cell layer of anther epidermis (Evrard *et al.*, 1991).

The different patterns of cellular expression and developmental regulation shown by PRPs probably correlate with their different structural properties. The absence of Tyr in wheat WPRP 1, like the low content of Tyr in the proline-rich domain of hybrid bean PvPRP 1, could be related to their respective absence of response or inhibition after wounding. The presence of Tyr in some repeats of other PRPs (Table 4) makes them likely sources of help in the rapid hardening of the wall, as a protection against environmental stresses by cross-linking of these pre-existing Tyr-containing wall proteins (Bradley *et al.*, 1992). This rapid response would take place before transcription activation of defence mechanisms. In PRPs, a positive response to wounding has been observed only for carrot p33 gene. For extensin it was proposed that the amino acid sequences YXY or Y_3 in their repeat elements would favour cross-links within and between proteins, and make extensins insoluble in the cell wall (Corbin *et al.*, 1987). The absence of these elements in carrot p33 protein as well as in the other PRP protein would limit their Tyr cross-linking to intermolecular interactions less extensive than the ones described for extensin proteins. Internal and external cross-links through cysteines in the cell wall could be possible for hybrid PRPs through the Cys present in their C-terminal hydrophobic domains once their hypothetical cell wall location had been demonstrated. It has to be taken into account that the distinct domains that these proteins show could also indicate a processing of the protein after deposition in the cell wall. In this case the proline-rich domain could act as a new type of targeting signal in plant cells. Moreover, the different content of basic and acid amino acids in PRPs gives them different net charges, which could allow different potential sites from ionic interactions with the cell components. These could be positively charged such as extensins or the same PRPs, and/or negatively charged such as pectins (Showalter & Rumeau, 1990).

IV. GLYCINE-RICH PROTEINS (GRPS)

1. *Occurrence of glycine in plant tissues*

In some plant tissues, where the hydroxyproline content in the cell wall is low, glycine is a major fraction of the total protein nitrogen (Varner & Cassab, 1986). These tissues include the soybean seed coat (Rackis *et al.*, 1961), containing 11% glycine; the gourd (*Cucurbita ficifolia*) seed coat, 21% Gly (Dreher *et al.*, 1980); the pumpkin (*Cucurbita pepo*) seed coat, where the major protein of the cell walls contains more than 47% Gly (Varner & Cassab, 1986); milkweed (*Periploca graeca*) stem cell walls, 31% Gly (Melin *et al.*, 1979); and oat (*Avena sativa*) coleoptile epidermal cell wall, 27% Gly (cited by Varner & Cassab, 1986). They are also present in the cell wall of more distant species such as *Chlamydomonas reinhardtii* cell walls, containing 23% Gly (Goodenough *et al.*, 1986); and *Thermomicrobium roseum*, a Gram-negative obligate thermophilic bacterium with a cell wall protein containing 34% Gly (Merkel, Durham & Perry, 1980). Although glycine-rich proteins are obviously a group distinct from proline-rich proteins, they are related in location in the cell wall, in a number of features of their expression and in their repetitive sequence. They have also been frequently cloned using probes for proline-rich proteins, due to the fact that the nucleotide triplet codings for glycine and proline are complementary.

Condit & Meagher (1986) were the first to isolate a gene (ptGRP1) from petunia plants which coded for a protein containing 67% of Gly. The protein sequence was highly repetitive, as expected for a structural cell wall protein with repeating units formed by the sequence $(Gly\text{-}X)_n$. It had a signal peptide indicating that it could be transported out of the cytoplasm. The glycine-rich repetitive region can be represented as GXGX, where X is either Gly

Table 5. *cDNA and genomic clones encoding glycine-rich proteins*

Name	Nature	Plant	aa	Reference
atGRP-1	Partial cDNA	*Arabidopsis*	210	De Oliveira *et al.* (1990)
atGRP-2	Complete cDNA	*Arabidopsis*	**203**	De Oliveira *et al.* (1990)
atGRP-3	Complete cDNA	*Arabidopsis*	**145**	De Oliveira *et al.* (1990)
atGRP-4	Partial cDNA	*Arabidopsis*	112	De Oliveira *et al.* (1990)
atGRP-5	Partial cDNA	*Arabidopsis*	173	De Oliveira *et al.* (1990)
GRP 1, 8	Genomic	Bean	**465**	Keller *et al.* (1988)
GRP 1, 0	Genomic	Bean	**252**	Keller *et al.* (1988)
ptGRP 1	Genomic	Petunia	**384**	Condit & Meagher (1986)
Class III-wM	Complete cDNA	Wounded tomato stem	**132**	Showalter *et al.* (1991)
Class III-wN	Partial cDNA	Wounded tomato stem	120	Showalter *et al.* (1991)
Class III-uE-7	Partial cDNA	Unwounded tomato stem	53	Showalter *et al.* (1991)
Class IV-w1-8	Partial cDNA	Wounded tomato stem	nd	Showalter *et al.* (1991)
Class IV-w10-1	Partial cDNA	Wounded tomato stem	nd	Showalter *et al.* (1991)
Class V-uA-3	Partial cDNA	Unwounded tomato stem	129	Showalter *et al.* (1991)
Class V-uK-4	Partial cDNA	Unwounded tomato stem	59	Showalter *et al.* (1991)
hvGRP	Genomic	Barley	**200**	Rohde *et al.* (1990)
Osgrp-1	Genomic	Rice	**165**	Lei & Wu (1991)

aa, Number of protein amino acids including the signal peptide; complete sequences are indicated in bold figures. nd, not determined.

Table 6. *Amino acid sequence repeats in GRPs*

Repeat	Number of repeats (name, plant)
G_n-X	30 (atGRP-2, *Arabidopsis*); 21 (atGRP-4, *Arabidopsis*); 30 (atGRP-5, *Arabidopsis*); nd (GRP 1.8, bean); nd (GRP 1.0, bean); 33 (hvGRP, barley); nd (Osgrp-1, rice)
$(G_nAG_n)_n$-F/H	19 (atGRP-1, *Arabidopsis*)
G_4N/RYQ	6 (atGRP-3, *Arabidopsis*)
G-X-G-X	nd (ptGRP-1, petunia)
GYGYGYG	3 (Osgrp-1, rice)
$G_{2\text{-}6}$-R	9 (class III-wM, tomato); 9 (Class III-wN, tomato); 6 (class III-uE-7, tomato)
$G_{2\text{-}6}$-YP	6 (class III-wM, tomato); 6 (class III-wN, tomato)
$SP_4SPSP_4Y_3K$ and $G_{2\text{-}6}$-R/Y-P	nd (class IV-w1-8, tomato); nd (class IV-w10-1, tomato)
$G_{2\text{-}5}$-R	11 (class V-uA-3, tomato); 6 (class V-uK-4, tomato)

A, Alanine; F, phenylalanine; G, glycine; H, histidine; K, lysine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; X, any amino acid; Y, tyrosine; nd, not determined. Amino acids that are equally frequent in a position are linked by a slash (/). References for cDNA and genomic clones are given in Table 5.

or one of the non-glycine residues in the region. A model for the mature protein was proposed. It is represented by a β-pleated sheet with 8 anti-parallel strands. Later, other genomic sequences corresponding to GRPs were isolated. For example, in bean a genomic clone was isolated (Keller, Sauer & Lamb, 1988), which contained two genes, GRP 1.8 and GRP 1.0, separated by 2·85 kb. In monocots two genes have been isolated from graminaceous species. The first one in barley (Rohde *et al.*, 1990) contained an intron interrupting the N-terminal region of the GRP coding sequence; it has some sequence similarity to vertebrate cytokeratins and Cys at the C-terminus. The second one, in rice, was named Osgrp-1 (Lei & Wu, 1991). Moreover, complete or partial cDNA sequences also corresponding to GRPs were isolated from total *Arabidopsis* cDNA libraries (de Oliveira *et al.*, 1990), from clones GRP 1–5 and from wounded or unwounded tomato stems' cDNA libraries (Showalter *et al.*, 1991). The last can be grouped in classes III, IV and V. A summary with the GRP clones described is presented in Table 5. Different repetitive motives have been identified from the isolated clones. As can be observed in Table 6, all of them agree with the consensus sequence G_n–X.

These glycine-rich proteins are related to the cell wall, have a repetitive structure, and have a signal peptide that may allow secretion out of the cell. Other proteins containing at least glycine-rich fragments have been published (Gómez *et al.*, 1988; Crétin & Puigdomènech, 1990). These proteins lack a signal peptide in their N-terminus; they contain repetitive motives (GGYGG) different from the ones described for wall proteins, and RNA-binding consensus sequences in the part of the sequence which is not rich in glycine. For these reasons it is thought that this second family of glycine-rich proteins can be cytosolic proteins, instead of wall components. The stretch containing a high amount of glycine may be important to interact with other cellular components which remain unknown.

The study of GRP proteins is less advanced compared with the data available for HRGPs or PRPs. Until now only one GRP has been isolated by salt extraction from the walls of strawberry fruits (Reddy & Poovaiah, 1987), although only its amino acid composition is available. The different studies of protein immunolocalization of GRPs in the cell walls of bean (Keller *et al.*, 1988), tomato, tobacco and petunia plants (Condit, McLean & Meagher, 1990; Ye & Varner, 1991) have therefore used antibodies raised against bean GRP 1.8 fusion protein or a synthetic peptide from the mature ptGRP.

2. *Immunolocalization*

Different approaches have related GRPs with the plant cell wall in different species. Antibodies raised against a fusion protein of β-galactosidase and bean GRP 1.8 allowed the detection of a protein of 53 kDa in a protein fraction extracted from cell walls of bean ovaries (Keller *et al.*, 1988). In a second approach, immuno tissue prints showed that the glycine-rich proteins were localized in the vessel elements, close to the inner epidermis of the pod wall of bean ovaries and in the inner side of the vascular ring of young bean hypocotyls (Keller *et al.*, 1988). A more accurate immunolocalization of GRPs in the vascular tissue of different bean organs was later done using the immunogold cytochemical localization method (Keller, Templeton & Lamb, 1989*b*). In young and old hypocotyls the protein was restricted to unlignified phloem (Keller *et al.*, 1989*b*; Ryser & Keller, 1992) and to tracheary elements of the protoxylem cells following a pattern very similar to wall lignification as determined by toluidine blue staining. In ovaries and seed coats of bean, GRPs were also associated with tracheary elements.

In young stems of soybean GRPs were also associated, by tissue prints, with the primary xylem and unlignified primary phloem. In older soybean stems, GRPs appeared associated with the secondary xylem, being gradually insolubilized in primary xylem and phloem. By immunogold cytochemical localization GRPs were observed in primary phloem and in primary and secondary xylem of young and old soybean stems and young petioles, and in protoxylem cell walls of soybean roots (Ye & Varner, 1991). In Solanaceae species such as tomato, tobacco and petunia, the same approach showed that GRPs were also localized in vessel elements of stem xylem (Ye *et al.*, 1991).

In petunia, an antibody raised against a synthetic peptide corresponding to the mature ptGRP 1 protein allowed the identification of a 23 kDa protein in young leaves. Tissue prints from petunia stems indicated that GRPs were localized in vascular tissue, especially to the phloem, and either in the epidermal cells or a layer of collenchyma cells directly below the epidermis (Condit *et al.*, 1990).

Recently, GRPs have been more precisely localized by immunoelectron microscopy (Ryser & Keller, 1992) in bean cell corners around young protoxylem and metaxylem vessels, in dictyosomes and endoplasmic reticulum of xylem parenchyma cells neighbouring protoxylem cells, and in the unlignified modified primary cell walls of the oldest protoxylem vessels. Although GRP and lignin deposition is parallel, the two processes have been shown to be independent (Keller *et al.*, 1989*b*; Ryser & Keller, 1992). These authors were unable to localize GRPs in the dictyosomes and endoplasmic reticulum of protoxylem cells and therefore suggested that GRPs are synthesized by xylem parenchyma cells. Then GRPs would be secreted to the primary cell walls of dead protoxylem vessels, and they might help to confer on the walls the elasticity needed during the elongation of the tracheary elements.

3. *Expression of GRP genes*

The genes coding for GRPs, like those coding for HRGP and PRP genes, are developmentally regulated. The petunia PtGRP gene was expressed mainly in stems, leaves and, to a lesser extent, in flowers, but never in roots (Condit & Meagher, 1986, 1987). Four related transcripts were identified by northern blotting analysis. They correspond to a transcript of 1·6 Knt and three more of 2·2, 1·7 and 1·2 Knt. All of them showed different organ-specific patterns of expression. The 2·2 Knt transcript was mainly expressed in flowers. In leaves the 1·6 Knt transcript, which corresponded to the probe employed, was induced within 5 min after wounding, and its maximal expression was achieved after 90 min. This enhancement of GRP 1 mRNA levels by wounding appears to be one of the earliest events of the plant wound response.

Tomato GRP class III transcripts were also shown to be actively induced after stem wounding locally

and systemically, whereas class V transcripts were reduced (Showalter *et al.*, 1991, 1992). Class III tomato GRP was expressed in stems after drought stress, ABA treatment and between 8 and 12 h after wounding. Instead Class V tomato GRP was expressed in stems and roots, decreasing in both tissues after wounding.

Bean GRP 1·8 kb gene was expressed only in young hypocotyls until 9 d after germination, in developing ovaries, and in roots (Keller *et al.*, 1988). Wounding studies by northern blotting analysis on young and old bean hypocotyls indicated an initial weak induction after 8 h in old hypocotyls. In both young and old hypocotyls gene expression was inhibited 12 h after wounding (Keller *et al.*, 1988).

Arabidopsis GRP were differently regulated. GRP 1 and GRP 2 genes were mainly expressed in roots, stems, leaves, seed pods and flowers, GRP 3 in stems and leaves, GRP 4 in leaves, stems and flowers and GRP 5 mainly in seed pods as well as in roots and stems. They also produced different responses to external stimuli. Salicylic acid first increased and then reduced GRP 1 transcripts, GRP 2 and GRP 5 response was retarded, while GRP 3 increased continuously with time. After drying, GRP 1 and GRP 2 mRNA disappeared, GRP 3 first increased its expression but then reduced it and GRP 5 was stable. Ethylene and ABA only stimulated GRP 3 moderately (de Oliveira *et al.*, 1990).

Rice Osgrp-1 encoded two transcripts (0·9 and 0·66 Knt) with different 5′ sites (Lei & Wu, 1991). Both transcripts were differently regulated, as happened for carrot HRGP (Chen & Varner, 1985*b*). The expression of the 0·66 Knt transcript increased gradually as the rice plants developed, whereas the 0·9 Knt transcript expression was not evident until the plant vascular system was in active differentiation.

In situ hybridization studies using GRP 1·8 bean probe (Ye & Varner, 1991) showed that in bean and soybean GRPs were expressed in all cells that were, or were going to become, lignified. In young soybean stems and hypocotyls, GRPs were highly expressed in the primary xylem and also in the primary phloem. In older soybean stems they were expressed in young growing primary xylem cells and in newly differentiated secondary xylem cells, in the primary xylem of young soybean petioles, and in the vascular seedcoat tissues. In bean petioles and stems GRPs were also always associated with primary xylem structures (Ye & Varner, 1991). Similar patterns of expression were found in tomato petioles and stems, and in petunia and tobacco leaves and stems (Ye *et al.*, 1991). In conclusion, GRP genes have been shown to be developmentally regulated. They are either induced or repressed after wounding, depending on the specific gene observed. They do not appear strongly responsive to ethylene and ABA, but they are in some cases sensitive to drought.

4. *GRP regulatory sequences*

Some studies have been reported in order to define the regulatory elements present in the promoter of the bean GRP 1·8 kb gene. A sequence of 494 bp in the promoter region is required to direct the correct expression of the gene in tobacco-transformed plants (Keller, Schmid & Lamb, 1989*a*). Expression appeared in roots, stems, leaves and flowers. It was induced in young stems 30 min after wounding. This induction was faster than the one described in old bean hypocotyls by northern studies. Only a small set of cells, inside the vascular cylinder in pith parenchyma cells, participated in the GRP wounding response. Roots, like stems, also responded to wounding, inducing GRPs in the region adjacent to the damage surface. Later, a more detailed study (Keller & Baumgartner, 1991) using promoter deletions, established four regulatory elements in the promoter of the bean GRP 1·8 kb: SE1, stem expression regulatory element (−121, −94); RSE, root expression regulatory element (−94, −76), which is independent of other sequences; SE2, stem expression regulatory element when helped by RSE (−293, −205); NRE, negative regulatory element which allows expression only in vascular tissues and suppresses expression in all other tissues (−199, −186).

In these studies vascular specific expression of GRP 1·8 gene promoter has been found to be controlled through negative and positive interactions between cis-acting regulatory regions. When they are altered it gives an anomalous pattern of gene expression.

V. CONCLUDING REMARKS

The analysis of the components of the plant cell wall has yielded an impressive amount of information on the carbohydrates that form the largest part of it. This information has enabled the proposal of a number of models for the structure of this essential compartment of plant cells (Carpita & Gibeaut, 1993). Proteins were rarely taken into account in these models until extensins were discovered. Since the early eighties, the application of recombinant DNA methodologies to the study of plant cell wall components has produced an increasing number of available cloned sequences, corresponding essentially to what appear to be the main structural proteins of this characteristic compartment of plant cells. The available clones correspond to highly repetitive proteins containing a high proportion of either proline or glycine in the whole or part of their sequence. These proteins can be classified in large groups such as HRGP, PRP or GRP, and in subgroups within these ones, depending on the features of their sequence and on the patterns of expression observed. In fact, it has not been proved

that all the proline-rich or glycine-rich sequences already characterized in plants are components of the cell wall, and in some cases it is probable that they are located elsewhere. An example of this situation is provided by one of the subgroups of glycine-rich proteins that has been shown to be an RNA-binding protein, and probably cytoplasmatic, or HyPRPs, a group of PRPs, where part of the sequence might be processed and have a function of protection of the plant instead of structural. Also, a number of storage proteins, especially in cereals, have proline-rich stretches and are transported and stored in protein bodies. It is also very probable that the data now available only correspond to a small proportion of the structural components of the cell wall. Further work in this direction is necessary to construct better and more complex models.

The essential repetitive elements of the proline-rich proteins are characteristic of each type of protein and each plant species. In most of the dicotyledonous plants, the SPPPP sequence is the essential elementary repeat of HRGPs, although it might be accompanied by other motives specific for each protein. In some cases the SPPPP sequence appears to have degenerated (see Tables 1 and 2). The repetitive nature of these proteins constitutes a very interesting model for the evolution of repetitive proteins. In species where a number of genes code for similar proteins, the similarity of sequences within a given species may indicate either that the duplications have occurred after the divergence of the species, or that a process of homologous recombination has been acting on these genes, producing the homogenization of the sequences. In some cases, for instance in maize, these mechanisms might have occurred even within a given gene, producing a high polymorphism in the size of the protein and the corresponding mRNA. The SPPPP element is not the essential protein repeat, at least in the HRGPs from graminaceous species that have been analyzed so far, although it might be present once in some of the available sequences. PRPs have, in general, more heterogeneous repeating elements, at least when comparing one species to another one, although it is not certain that this group of sequences includes proteins having identical functions in the plant.

While an increasing number of proline-rich or glycine-rich cDNA or genomic sequences have been cloned, only a small number of protein species has been purified. Therefore, structural information about these proteins is very scarce. This information should be very useful in constructing models for the interaction of the protein with other components of the cell wall and with other elements of the cell, in particular in the plasma membrane or across it. There is also very limited information about the process of modification, transport and polymerization in the cell wall, and its relation to defined physiological stages of cell growth and defence. Data on the sequences that determine the transport and the interaction of cell wall proteins with other cell wall components, including themselves, are beginning to appear, and they are essential to understanding the dynamic processes that govern cell division and cell elongation. In this sense it is obvious that the relations of these proteins with the carbohydrate components of the cell wall are obligate (and mostly unknown) data in understanding how the wall is built.

The genes coding for proline-rich and glycine-rich proteins have well-defined patterns of expression. These can be divided into two types: control in defined stages of plant development and induction by defence responses. In both cases, these features indicate that the genes coding for these structural proteins are interesting markers for processes which are essential to understand plant growth and survival. In development, it has been shown in some cases that synthesis of these proteins occurs early in the formation of the cell plate. Both protein and RNA probes coding for HRGPs and other cell wall glycoproteins have been shown to be useful markers for tissues active in cell division, and GRP probes appear to mark the formation of xylem. PRP genes are expressed, at least in soybean, in a number of precise steps in the formation of the developing plantlet. The proline-rich nodulins are among the most interesting markers in the formation of nodules. Genes coding for proline-rich or glycine-rich proteins show a specific expression in developing flower organs, such as anthers or pistils, and they may take part in the compatibility reactions occurring in the pistil.

The analysis of promoters corresponding to these genes may be a source of elements important for gene constructions having interesting induction properties, as is the case in the tapetum or the silk. Consequently, these promoters may be very useful in transgenic plants where a specific product has to be directed to a defined cell type. The identification of transcription factors related to these promoter elements may be possible once these promoters have been analyzed. Results in this direction are beginning to appear, and these studies may help us to understand regulatory pathways in plant development or defence.

It is probable that the functions of the proteins here described are very different. This is the case, for instance, for nodulins, which are supposed to take part in the formation of the symbiotic nodules in legumes. However, precise information on specific functions of any of these proteins is lacking. Molecular techniques have enabled detection of the mRNAs in precise cell types, and these results allow speculation about the involvement of the proteins in specific cellular processes. Roles for HRGPs have also been discussed in the light of general models for

the cell wall (Carpita & Gibeaut, 1993); however, they still contain a large degree of speculation. The recent findings showing a rapid polymerization of proline-rich proteins upon wounding indicate a specific function in creating a tightly bound wall in stress conditions (Bradley *et al.*, 1992). Final elucidation of the function of these proteins in relation to the other components in the wall and in relation to the other components that transduce the information from the cell through the membrane are challenging questions for the near future.

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