Glycine-rich proteins as structural components of plant cell walls

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Abstract. Glycine-rich proteins (GRPs) have been found in the cell walls of many higher plants and form a third group of structural protein components of the wall in addition to extensins and proline-rich proteins. The primary sequences of GRPs contain more than 60% glycine. GRPs are localized mainly in the vascular tissue of the plant, and their coding genes provide an excellent system to analyze the molecular basis of vascular-specific gene expression. In French bean, the major cell wall GRP has been localized at the ultrastructural level in the modified primary cell wall of protoxylem. Immunological studies showed that it forms a major part of these highly extensible and specialized cell walls. Specific digestion of GRP1.8 from bean by collagenase suggests that it shares structural similarities with collagen. The protein is synthesized by living protoxylem cells as well as xylem parenchyma cells. After cell death, GRPs are exported from neighboring xylem parenchyma cells to the protoxylem wall, a rare example of protein transport between cells in plants. We propose that GRPs are part of a repair system of the plant during the stretching phase of protoxylem.

Key words. Cell wall; glycine-rich protein; protoxylem; structural protein; vascular tissue; xylem.

Introduction

GRPs form a class of structural cell wall proteins with very characteristic primary amino acid sequences. In our review we will focus on proteins which have been characterized as cell wall proteins either by (i) ultrastructural immunolocalization or (ii) the presence of a signal peptide sequence in the coding region of the corresponding gene indicating the export of the protein to the apoplastic space. Other glycine-rich proteins (frequently intracellular, RNA-binding proteins) will not be considered in this review. Structural cell wall GRPs have glycine contents up to 60 or 70% of all amino acid residues. This is a very high percentage compared with a glycine content of 5% in globular proteins such as hemoglobin or even compared with some glycine-rich structural proteins in animals. For example, in collagen, which is the major protein of the connective tissue, about one-third of all amino acids are glycine. However, there are proteins in animals which are as glycine-rich as the plant GRPs. Among these is loricrin, a cell envelope protein in mammals which contains 55.1% glycine (but also many cysteine residues which are rare in plant GRPs) [1]. Mutations in loricrin can result in serious diseases in humans [2]. In addition, spider silk fibroin (47.3% gly, [3]) and an eggshell protein of the human parasite Schistosoma mansoni (44% gly, [4]) are very glycine-rich. Obviously, GRPs have biochemical properties which contribute to the strengthening of biological structures or which allow the formation of very tensile fibers. These specific properties of GRPs were used for different purposes by many different organisms in the evolution of plants and animals.

Although less work has been done on the plant cell wall GRPs than on the two other major classes of structural cell wall proteins (extensins and PRPs), considerable information has accumulated on the type and variability of sequences of GRP structural proteins in different species. In addition, several authors have found a highly cell-type specific expression of glycine-rich proteins, indicating that these proteins are not part of all cell walls but instead must be involved in conferring specific properties to some cell walls only. In the species studied, GRPs are ex-

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pressed only in a small number of cells, particularly in cells of the xylem tissue. A detailed, ultrastructural localization has been determined for the French bean GRP1.8 in the xylem tissue. GRPs have been found in all species of higher plants where they have been looked for. This suggests that they are involved in essential functions for the plant but there are no studies yet which provide clear evidence for the functional role of GRPs. The detailed immunolocalization studies on the bean GRP1.8 have resulted in a hypothesis of the function of the xylem-specific GRPs [5, 6]. Here we will give an overview on the primary structure, gene expression and immunolocalization of GRPs in higher plants.

The primary structure of GRPs

GRPs are characterized by their high content of glycine residues. GRPs, however, are not necessarily structural proteins, as RNA-binding proteins also have glycine-rich domains [7–9]. Since this article is focused on structural GRPs localized in the cell wall, only proteins are included for which extracellular localization has been shown or is proposed. Export of the protein from the cell has been shown for ptGRP1 and GRP1.8, two glycine-rich proteins of petunia and bean, respectively [5, 10]. For other GRPs, extracellular localization is proposed by a predicted N-terminal peptide for export of the proteins [11].

grp genes were isolated from a broad spectrum of dicotyledonous and monocotyledonous plants by molecular biological tools such as screening of complementary DNA (cDNA)- or genomic DNA libraries or by differential screening. In addition, anti-ptGRP1 antibody used in immunoblot experiments with protein extracts of different mono- and dicotyledonous plants detected one or several bands, suggesting the presence of ptGRP1 homologues in these plants [12]. The isolation and detection of *grp* genes and GRPs in very different plants suggests that this class of structural proteins is common in many if not all higher plants.

Some GRPs consist almost exclusively of glycine-rich sequences, e.g. ptGRP1, GRP1.8 and GRP1.0 of bean, OsGRP1 of rice, atGRP-5 of *Arabidopsis*, GRP-22 of *Brassica napus* or zmGRP3 of maize [13–18], whereas others have glycine-rich domains adjacent to domains with lower levels of glycine such as atGRP-3 and atGRP-6 of *Arabidopsis*, TFM5 of tomato or OI14-3 and OI2-2 of saltbush [16, 19–21]. A third group are GRPs that have an increased glycine content but not domains that are particularly glycine-rich. Such proteins are e.g. the ENOD-GRPs that are expressed in nodules of *Vicia faba* [22, 23]. The first group of GRPs has an overall glycine content of up to 70% and is therefore easily identified as a member of the GRP class of structural proteins. The classification of other proteins as GRPs based on the presence of a

glycine-rich domain is somewhat arbitrary and depends on the size of the glycine-rich domain and the actual glycine content.

The GRP protein sequence often follows the motif $(Gly-X)_n$ in the glycine-rich region where X is often glycine, but can also be another amino acid. Ala, Ser, Val, His, Phe, Tyr and Glu are common at the X position, but other amino acids are also possible. The $(Gly-X)_n$ motif is not very often interrupted and after such an interruption often immediately resumes. In some cases the motif varies, e.g. $(G_2-X)_n$ [24], or is more complex, e.g. $(G_4-H-G_2-H-G_4)_n$ [25]. The ENOD-GRPs that do not have glycine-rich domains but rather a generally increased glycine content do not contain particular repetitive motifs [22, 23]. A list of repetitive motifs of GRPs is shown in table 1. Beside the $(G-X)_n$ motif, higher-order repetitive sequences that are rarely perfect are sometimes found and were proposed to be important for the formation of the secondary structure of the protein (see below). ptGRP1 contains two different families of repeats of about 40 amino acids. The F1 family consists of two repeats, whereas the F2 family is repeated five times [13]. In OsGRP1, 21–32 amino acids are repeated four times [15]. Almost perfect higher-order repeats are found in GRP-22 of *Brassica napus* where 42 amino acids are repeated three times [17] and in

Table 1. Summary of repetitive motifs in the primary sequence of extracellular GRPs. Only GRP proteins with a predicted N-terminal signal peptide [11] or an experimentally demonstrated extracellular localization are included. The amino acid sequence motifs of the proteins are indicated in the single letter code, with X being any amino acid.

Organism	Gene	Repetitive amino acid motif	Reference
Petunia	*ptgrp1*	$(G-X)_n$	13
Bean	*grp1.0*	$(G-X)_n$	14
	grp1.8	$(G-X)_n$	14
Red goosefoot	*HC1*	$(G_3-Y-H-N-G_4-Y-N-N)_{10}$	37
Arabidopsis	*atGRP-3*	$(G_4-N-Y-Q)_3$	
		$(G_4-R-Y-Q)_3$	16
	atGRP-5	$(G_{3-5}-X)_n$	16
	atGRP-6	$(G_2-A-S-G_2)_3$	
		$(G_2-A-S-G_3-P)_3$	19
Barley	*hvgrp*	$(G-X)_n$	66
Rice	*Osgrp1*	$(G-X)_n$	15
Tobacco	*tgGRP*	$(G_{2-7}-X)_n$	41
Alfalfa	*MsaciA*	$(G_{3-4}-Y-X_{1-2})_6$	
		$(G_4-H-G_2-H-G_4)_2$	25
Carrot	*CEM6*	$(G_2-X)_n$	24
Brassica napus	*GRP-22*	$(G-X)_n$	17
Pinus taeda	*pLP5*	$(G-X)_n$	42
Alnus glutinosa	*Ag164*	$(G-X)_n$	38
Saltbush	*OI 2-2*	$(G-H-G_3-Y)_7$	21
	OI 14-3	$(G-H-G_3-Y)_9$	21
Tomato	*Tfm-5*	$(S-G_{4-6}-S-G)_8$	20
Maize	*zmGRP3*	$(G_{3-10}-X)_n$	18
Wheat	*wgrp1*	$(G_3-F-G-A-G_3)_6$	
		$(G_3-F-G_3-A-G-A)_3$	26

Table 2. Summary of higher-order repetitive motifs of extracellular GRPs.

Protein	Higher-order repetitive amino acid motif	Number of repetitions
GRP1.8	EH(G)$_3$G/A(G)$_3$Q(G)$_3$A(G)$_3$YG/AAG/VG	6
GRP-22	GY(G)$_4$A(G)$_2$H(G)$_5$SGG/S(G)$_5$A(G)$_2$AH (G)$_3$Y(G)$_3$EGAGA(G)$_2$	3

The higher-order repeats of two GRPs, GRP1.8 of bean and GRP-22 of *Brassica napus,* are shown [14, 17]. The repeats in these proteins are almost perfect. In cases of inconsistency in the repeats, the different amino acids at the corresponding position are both indicated. The single letter code for the amino acids is used.

GRP1.8 where 22 amino acids are repeated six times [14] (table 2).

Based on the primary sequence of a GRP, a hydropathy profile can give valuable information regarding the chemical property of the protein. The difference in the number of nonglycine residues in a glycine-rich domain and the nature of these amino acids largely determines the profile. Thus, some GRPs are predicted to be hydrophobic, whereas others are rather hydrophilic. A correlation is found between the presence of Tyr instead of Phe and the hydropathy profiles of the proteins. ptGRP1, atGRP-5 and the wheat GRP wGRP1 do not contain any Tyr (except in the N-terminal signal peptide) [13, 16, 26], and their profiles indicate large hydrophobic regions (fig. 1A–C). In contrast, GRPs containing Tyr such as GPR1.8, atGRP-3 and OsGRP1 [14–16] show a rather hydrophilic profile (fig. 1D–F). To determine whether there is a direct correlation between Tyr versus Phe residues and the hydropathy profile, an analysis was made with modified ptGRP1, atGRP-5 and wGRP1 in which all the Phe residues were replaced by Tyr. The hydrophobic regions in these proteins were still present, indicating that Phe/Tyr residues alone are not the determinants of the hydropathy profile. Correspondingly, replacement of all Tyr residues by Phe in GRP1.8, atGRP-3 and osGRP1 does not render these proteins hydrophobic. Thus, the presence of Phe or Tyr and the hydropathy profile of GRPs correlate independently. This points towards basic differences in the function of the two different types of GRPs in the extracellular matrix. This view is supported by the subcellular localization of ptGRP1 and GRP1.8, respectively. Whereas ptGRP1 is localized at the plasma membrane/cell wall interface [27], GRP1.8 is found in the modified primary cell wall of protoxylem elements in bean hypocotyls and thus is not membrane associated [5]. In later stages of hypocotyl development GRP1.8 most likely becomes covalently cross-linked in the cell wall. GRP1.8 cannot be extracted from cell walls of older tissue, but in situ immunolocalization is still possible [28]. In the case of extensins, covalent cross-linking of proteins correlates with increased formation of (iso-) dityrosines [29–31]. Although the mechanisms of cross-

linking of GRP1.8 and possibly other GRPs is not known at this point, it is quite possible that by analogy, GRPs are also cross-linked in the cell wall through the formation of ether linkages involving Tyr [32]. As a consequence, Tyr residues might be important for the cross-linking of GRP1.8 and hence the function of the protein in the cell wall matrix.

The secondary structure of GRPs

The repetitive nature of the glycine-rich domains is likely to allow the formation of a β-pleated sheet structure. The higher-order repeats of ptGRP1, GRP1.8, OsGRP1 and GRP-22 mentioned above are thought to align as antiparallel strands that allow the formation of a β-pleated sheet [13–15, 17]. As an example, a scheme of the β-sheet structure formed by the antiparallel strands of OsGRP1 of rice [15] is shown in figure 2. The analysis of different GRPs listed in table 1 by a program predicting secondary structures [33] confirms this hypothesis (data not shown). Replacement of Gly by Ser or Ala still allows the formation of the ß-pleated sheet structure. Thus, these two amino acids in the X position of the (G-X)$_n$ motif do not interfere with the formation of such a structure. In some GRPs, amino acids with charged side chains are almost always at the odd position [13, 14, 16, 17]. Thus, in a β-sheet structure these sidechains project in the same direction from the β-sheet, leaving a large surface of hydrophobic property to the other side. This proposed structure would lead to a protein with a two-sided structure of which the two sides have different abilities of interaction with other compounds in the cell wall. With this proposed structure, even GRPs with rather hydrophilic hydropathy profiles might be able to establish hydrophobic interactions in the cell wall [13]. For GRP1.8, hydrophobic interactions in the cell wall matrix were indeed observed. Protein extraction experiments using different extraction conditions revealed that hydrophobic compounds are necessary to entirely remove soluble GRP1.8 from the extracellular matrix [26].

In the future, it will be necessary to study the secondary structure of GRPs in more detail. Matsui and co-workers [34] have investigated the structure of a GRP extracted from the aleurone layer of soybean by CD (circular dichroism) analysis using purified GRP protein and were not able to confirm a β-sheet structure. The exact primary sequence of this GRP is not known, though. In addition, this particular GRP seems to be glycosylated, which might interfere with the formation of a β sheet [34]. Thus, these data are not sufficient to allow for a determination of the general secondary structure of GRPs. In support of a β-sheet structure of GRPs, we have recently found a strong increase of β-sheet structure in a GST-GRP1.8 fusion protein overproduced in *Escherichia coli* compared
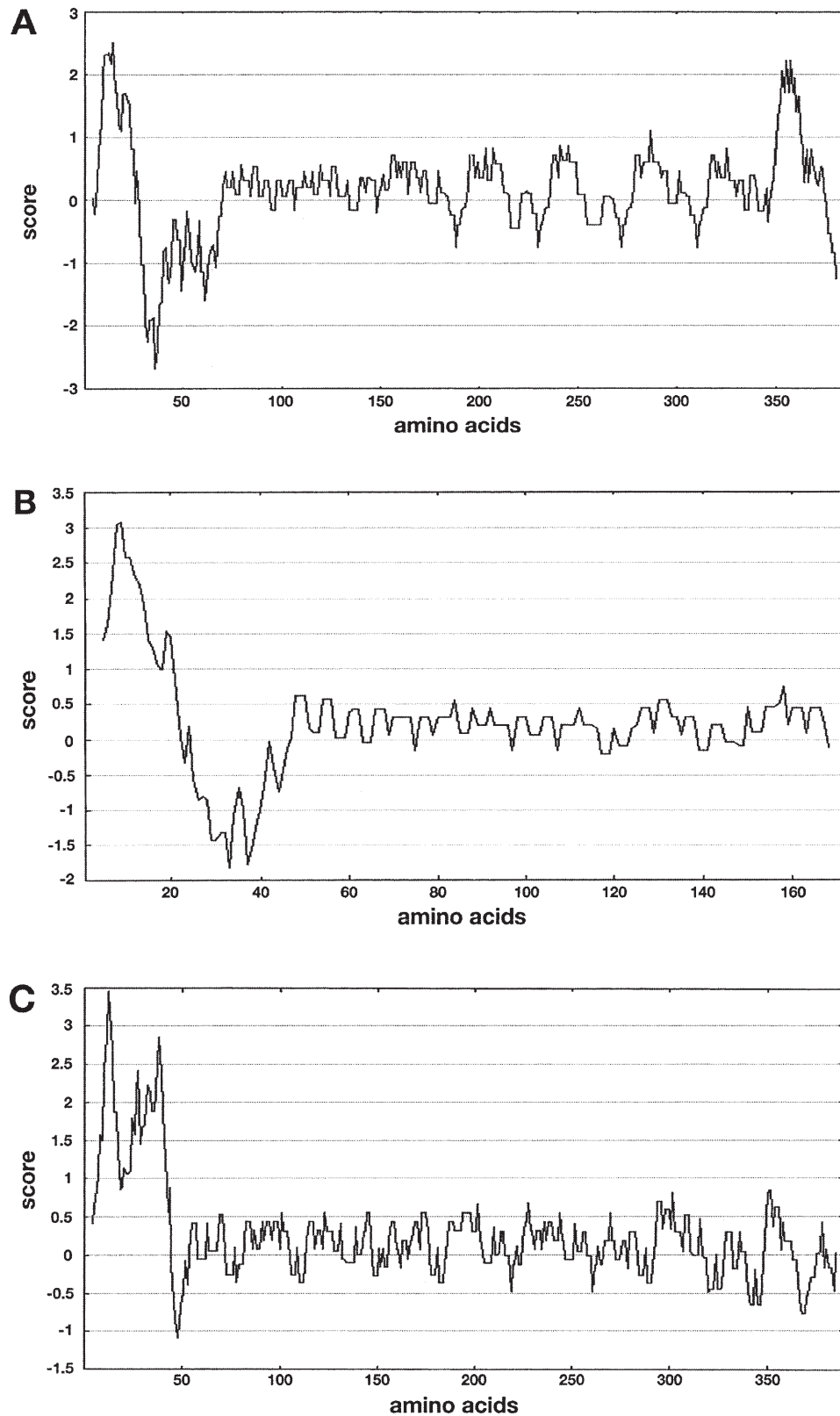
Figure 1. Hydropathy profiles of different GRPs. (*A – C*) Profiles of ptGRP1, atGRP-3 and wGRP1, three GRPs containing Phe and no Tyr in the glycine-rich domain.
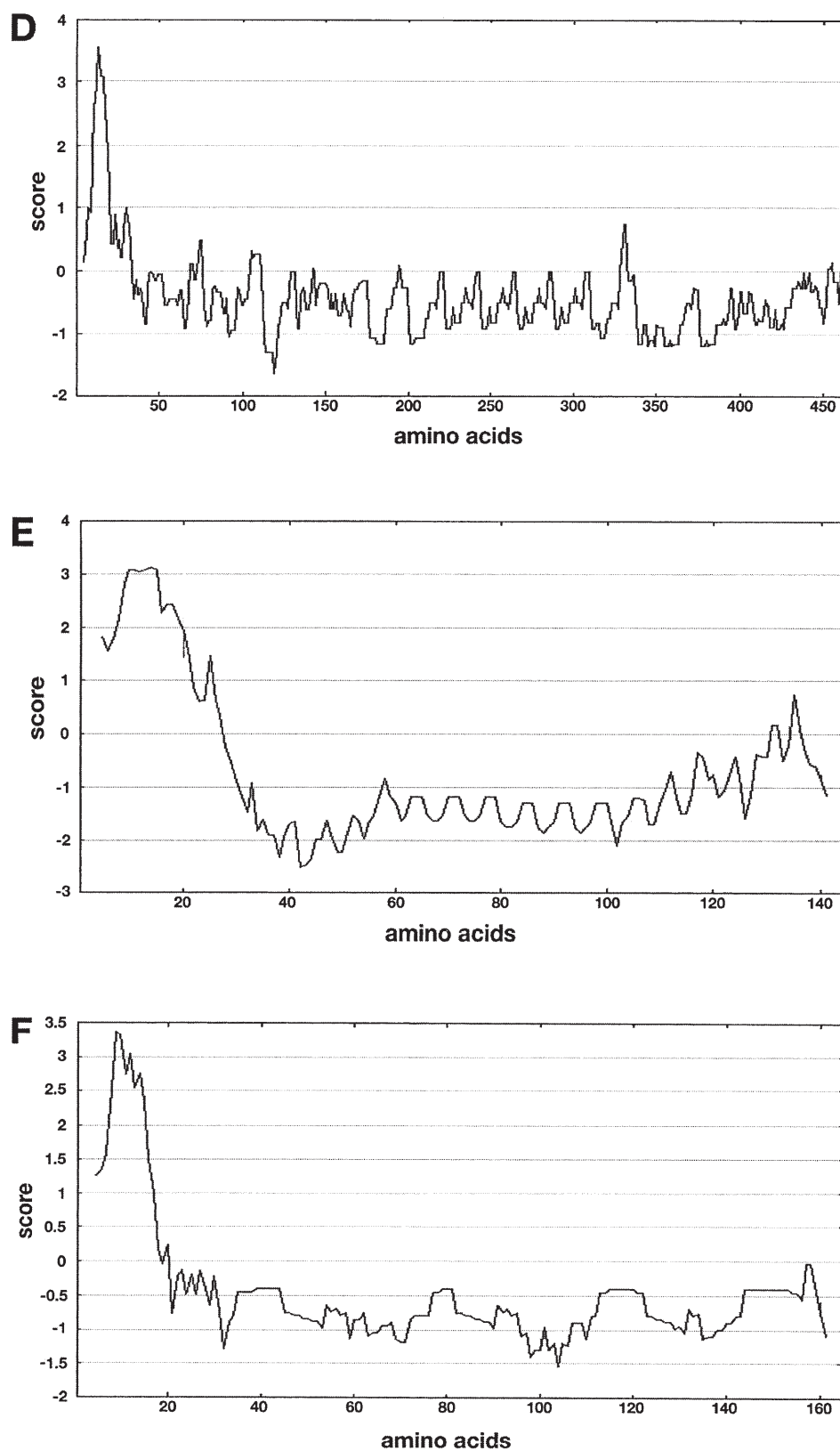
Figure 1 (continued). (*D–F*) Profiles of GRP1.8, at GRP-5 and OsGRP1, three GRPs containing Tyr and no Phe in the glycine-rich domain. The hydropathy profiles were established according to Kyte and Doolittle [67].

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    ARVIKYN...S..............N.S.S.S.Y.
                                         Y
 .Y.S.S.S.N...........S.Q...SQ..AK.Y.
Y.Y.Q.N..AQ.Q.S.............S.Q.S.S.Y.
                                         Y
    EHK.UY.S................K.Y.
                                    Y
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Figure 2. Antiparallel strands of the OsGRP1 form the putative β-sheet structure of the protein. The amino acids of the higher-order repeat found in OsGRP1 form the four antiparallel strands. The three (G-Y)$_3$ repeats are at the outer edge of the sheet structure and form the turns between two strands. The amino acids are shown in the single letter code. Dots represent glycine residues. Model according to Lei and Wu [15].

with the GST domain alone [Hauf G. and Keller B., unpublished data].

Specific degradation of GRP1.8 by collagenase

An interesting observation possibly relating to specific characteristics of the secondary structure of GRP1.8 was made using protease digests and immunolabeling. The labeling with specific antibodies against GRP1.8 on thin sections was strongly reduced after proteinase K digestion. The same observation was made after digestion with collagenase, an enzyme which is specifically cleaving the PXGP amino acid sequence. This sequence is not present in GRP1.8, and it is possible that secondary or tertiary structures are responsible for cleavage by collagenase. Specific digestion of the GRP1.8 protein domain by collagenase was also observed in several fusion proteins of GRP1.8 with glutathione-*S*-transferase (GST) [6]. The GST domain of the fusion proteins was not affected by collagenase digestion, whereas the GRP domain was completely digested. These results suggest that GRP1.8 has a secondary structure which is very similar to collagen, mimicking the cleavage site. Thus, GRP1.8 and collagen might be structurally similar, even if the primary amino acid sequences differ considerably.

Regulation of gene expression

Detailed studies of the expression patterns of *grp* genes revealed tissue-specific localization and developmental regulation of gene expression. Genes encoding GRPs such as *grp1.8* or *ptgrp1* are mainly expressed in vascular tissue. Immunolocalization with an anti-GRP1.8 antibody in bean hypocotyl localized the protein in xylem elements [5, 14, 28]. The second *grp* gene of bean, *grp1.0*, appears to be expressed in vascular tissue as well. This is suggested by the analysis of GUS expression driven by the *grp1.0* promoter in transgenic tobacco (fig. 3) [B. Keller, unpublished data]. The expression pattern of *grp1.8*-like gene(s) was studied in tomato, tobacco and petunia by in situ hybridization using the *grp1.8* coding

region as a probe. These experiments revealed expression of *grp1.8*-like gene(s) in xylem tissue and cambial cells of the stem [35]. Vascular expression was also found in hypocotyl, stem and petioles of soybean [36] by in situ hybridization using the same *grp1.8* probe. ptGRP1 is mainly localized in phloem tissue of stems and leaves of petunia, but is also detectable in older protoxylem as revealed by immunodetection in petunia [27]. Anti-ptGRP1 antibody used in immunolocalization experiments in rape and barley also revealed a signal in the vascular tissue [12]. Thus, in summary, it can be concluded that genes
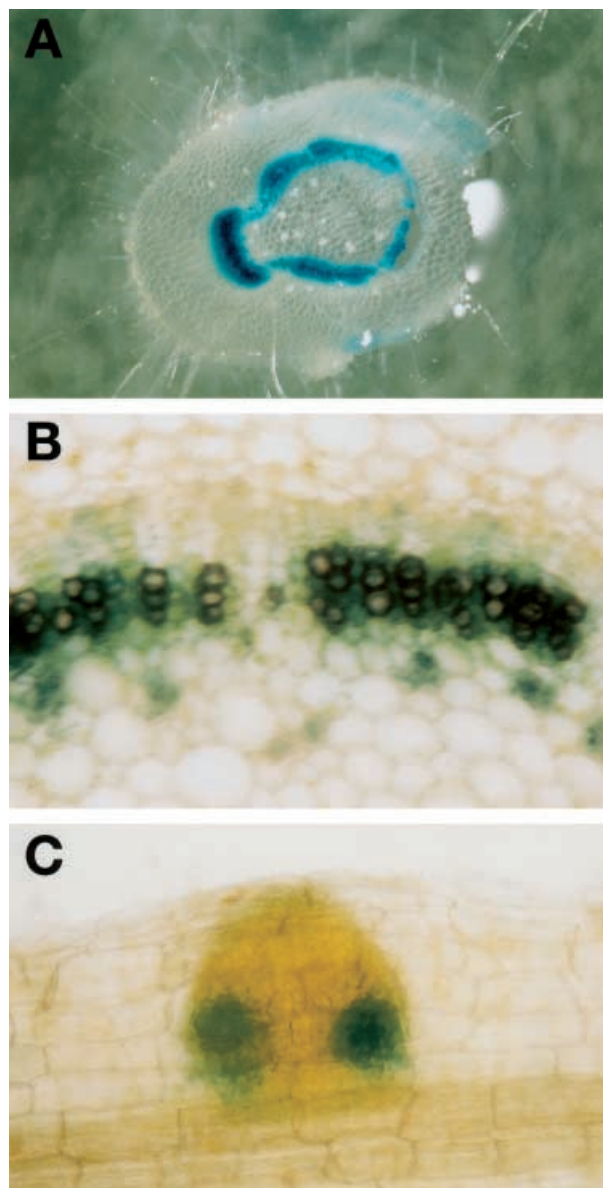

Figure 3. The bean *grp10* promoter confers vascular specific gene expression. A 430-bp fragment of the *grp10* promoter was translationally fused to the β-glucuronidase reporter gene (GUS). Expression was observed in the vascular tissue of young tobacco transformants in stems (*A, B*) and during the vascular development in newly forming lateral roots (*C*).

homologous to *grp1.8* or *ptgrp1* are predominantly expressed in vascular tissue.

In some cases *grp* expression has been shown to be developmentally regulated. *ptgrp1* as well as the *grp1.8* and *grp1.8*-like gene(s) are mainly expressed in young tissue, whereas the expression in older stages of development is strongly reduced [14, 27, 35]. Other *grp* genes are predominantly expressed in developing tomato fruits [20], during root nodule formation in *Vicia faba* and *Alnus glutinosa* [23, 37, 38], or somatic embryogenesis in carrot [24]. Besides developmental regulation, expression of *grp* genes has been shown to be altered by external influences such as wounding, hormone treatment, low temperature and water or ozone stress [14, 16, 17, 21, 22, 25, 39–41]. In fact, several *grp* genes were cloned in differential hybridization experiments performed with the goal to isolate genes induced by drought, water or low-temperature stress [21, 25, 42]. Thus, GRPs are not only required in certain stages of development but are often also stress-induced proteins.

The analysis of promoter *gus* fusion constructs in transgenic plants is a convenient way to study promoter activity and consequently gene expression patterns. The bean *grp1.8* promoter was analyzed in detail in this way. Tobacco plants transformed with a *grp1.8* promoter *gus* fusion construct exhibit GUS activity specifically in vascular tissue, confirming the vascular-specific localization of GRP1.8 [43]. The relatively strong *gus* expression conferred by the *grp1.8* promoter made it a good candidate to identify cis-elements present in the promoter. To this end, promoter deletion experiments were performed. The monitoring of changes in GUS expression patterns allowed the attribution of regulatory functions of a particular deleted sequence. Different cis-elements were identified that are important for expression of the *grp1.8* gene in the stem, roots and vascular tissue, respectively. An overview of these elements is shown in figure 4. While some cis-elements have a direct effect on transcriptional

activity of the promoter, others require interaction with a second cis-element present. Thus, combinatorial activity of the elements is necessary for the correct regulation of gene expression. Different elements are important for gene expression in stems (SE1 and SE2), roots (RSE) and vascular tissue in general (VSE). The activity of SE2 depends on the presence of RSE, as deleting RSE abolishes gene expression in stems driven by SE2 alone. Whereas SE1 is required for expression in stem, NRE (negative regulatory element) restricts this expression to the vascular tissue. Deletion of NRE leads to GUS expression in cortical cells and a reduced expression in the vascular tissue. Thus, NRE has both a negative, restricting, as well as a positive, activating function. The principe of negative regulation of vascular-specific gene expression as found with NRE also applies for other genes that are expressed in the vascular tissue [44, 45]. Further analysis revealed the presence of a positively acting element *vs-1* that partially overlaps with NRE. *vs-1* confers strong vascular-specific expression to a *grp1.8* or a CaMV 35S minimal promoter in transgenic tobacco [46]. Detailed analysis of the *vs-1* element through protein-DNA interaction studies showed that the bZIP transcription factor VSF-1 (vascular specificity factor 1) binds *vs-1* in vitro and stimulates transcription of a reporter gene in a *vs-1*-dependent manner in tobacco protoplast transfection experiments [46]. The VSF-1 binding site on *vs-1* lacks an ACGT, the typical palindromic core sequence bound by bZIP transcription factors [47, 48]. Furthermore, the binding site is not palindromic, which is very unusual for a bZIP transcription factor binding site [48]. The finding that the *vsf-1* gene itself is expressed in the vascular tissue and the considerable homology of VSF-1 to the rice bZIP transcription factor RF2a that is important for vascular development [48, 49] supports the hypothesis that VSF-1 is involved in the regulation of vascular gene expression.

Sequence comparison of different vascular-specific promoters can reveal conserved sequences, which suggests a
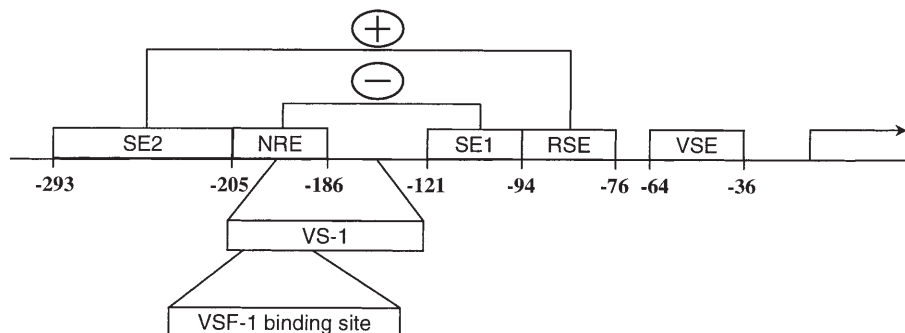

Figure 4. cis-elements of the *grp1.8* promoter identified through promoter deletion experiments. Positions in the promoter are given relative to the transcription start site indicated by the arrow. SE1 and SE2 confer activity to the promoter in the stem. RSE is required for root-specific expression, and VSE confers vascular expression. For its activity, SE2 requires interaction with RSE. The negative regulatory element NRE restricts the expression in the stem to the vascular tissue and therefore acts negatively on SE1, which confers general gene expression in the stem. *vs-1* overlaps with NRE and confers vascular expression. The binding site of the bZIP transcription factor VSF-1 on *vs-1* is in the 5′ half of *vs-1* and thus lies within NRE.

similar function as regulatory elements. Loopstra and Sederoff [50] cloned two xylem-specific genes of loblolly pine which both harbor in their promoters a 7-bp sequence that is also present in the promoters of *grp1.8* and *grp1.0* of bean [14]. These 7 bp are part of the NRE element of the *grp1.8* promoter and are thus potentially important for the restriction of gene expression to the vascular tissue.

Localization of GRPs in the cell wall

To learn more about the function of GRPs, their cellular and subcellular location was studied using immunocytochemistry in several studies [5, 6, 27, 43]. GRPs were consistently found to be associated with the protoxylem. Therefore, the development of this tissue will be described in some detail in the next paragraph.

Protoxylem cells are the first xylem elements differentiating from the procambium and are characterized in elongating plant organs by lignified annular and helical secondary-wall thickenings. The primary walls remain unlignified and will be partially degraded by hydrolases later in protoxylem development [51]. The mature protoxylem elements functioning in water and solute transport are dead cells. The crucial events of programmed cell death, such as vacuolar burst, degradation of the nucleus and cytoplasmic constitutents have been studied mainly in Zinnia mesophyll cells, differentiating into tracheary elements in vitro [52–55]. DNAses, RNAses and proteinases of differentiating tracheary elements have been studied in detail. However, little is known about polysaccharidases, and it is not clear whether the hydrolysis of the primary walls is initiated by the vacuolar burst or occurs already earlier. Recently, evidence was presented indicating that the ubiquitin-proteasome pathway of proteolysis is required both for induction of tracheary element differentiation and for the progression of the program in committed cells [56]. In summary, the following main steps are involved in protoxylem development: (i) cell elongation and vacuolation, (ii) deposition and lignification of the specific secondary-wall thickenings, (iii) partial hydrolysis of the unlignified primary walls and programmed cell death, followed by (iv) a period of passive elongation of the empty water-conducting channels.

The lignified annular and helical secondary-wall thickenings probably protect the dead protoxylem elements against the compressive forces of the surrounding tissue [57], and the hydrolyzed primary walls are thought to be necessary for the passive elongation of the protoxylem elements by the growing xylem parenchyma cells [51]. Elongation factors of 10, 25 and 120 were determined for the protoxylem of different plant species, by measuring the displacement of the ring shaped cell wall thickenings [58], the elongation factors including a certain amount of growth of the living protoxylem cells. During periods of intense growth, the protoxylem elements finally collapse, and their function is taken over by younger protoxylem elements and finally, at the end of elongation growth, by the metaxylem.

The ultrastructural location of GRP1.8 in etiolated bean and soybean hypocotyls was studied with the indirect immunogold method, using polyclonal antibodies [5, 6]. The immunolabel was sensitive to collagenase a serine protease cleaving GRP1.8 but not other proteins such as peroxidase, bovine serum albumin or glutathione-S-transferase [6], suggesting that the antibodies really recognize epitopes in a glycine-rich protein. GRP1.8 accumulated in the corners of protoxylem cells with intact primary walls, in the hydrolyzed primary walls of the protoxylem, and in cell corners between xylem parenchyma cells. In older, nongrowing regions of the etiolated hypocotyls cell corners between proto- and metaxylem also became labeled [5, 6]. After chemical fixation GRP1.8 secretion was observed in xylem parenchyma, but not in protoxylem cells [5]. After cryofixation, followed by cryosubstitution, labeled Golgi complexes could also be visualized in the protoxylem. Some damage was observed in the fixed cells, occurring probably during the small time lapse between the excision of the tissue and cryofixation. GRP1.8 was secreted in xylem parenchyma and protoxylem cells already in the apical hook of the hypocotyl, and the secretion in xylem parenchyma cells probably continues beyond the time point of programmed cell death of the adjacent protoxylem elements [6]. However, the exact integration in time of GRP1.8 secretion and other events associated with the period of programmed cell death of the protoxylem elements still remains to be determined.

Interestingly, accumulation of a protein recognized by GRP1.8 antibodies was also observed in the cell walls of isolated Zinnia leaf mesophyll cells regenerating into tracheary elements [59]. The modified primary walls of such cells have a granular-particulate ultrastructure instead of the more fibrillar aspect of protoxylem elements differentiating in vivo and subjected to passive elongation [60].

It was consistently observed that the GRP1.8-containing walls mainly occur between two protoxylem elements [5, 6, 27]. They do not form a coherent interface between protoxylem elements and xylem parenchyma cells. Therefore, speculation that GRP1.8 may form waterproofing or antimicrobial cell wall layers, at the protoxylem-xylem parenchyma interface, is not supported by the observed subcellular location of the protein. Interestingly, the GRP1.8-labeled cell walls, together with some unlabeled cell corners, are intensely stained with the protein stain Coomassie blue, suggesting that the modified primary walls of the protoxylem elements contain unusually high concentrations of protein. Two components can be recognized in the cell walls labeled with

GRP1.8 antibodies: (i) fibrillar polysaccharides staining with silver proteinate after periodate oxidation [61], and (ii) an apparently amorphous material, preferentially labeled with GRP1.8 antibodies. These materials form a three-dimensional network, apparently interconnecting the ring-shaped secondary wall thickenings, as well as secondary wall thickenings and the middle lamella of xylem parenchyma cells [6].

The distribution of GRP1.8 immunolabel in the proto- and metaxylem of etiolated bean and soybean hypocotyls established that lignification and GRP deposition are not correlated events [5, 6]. Using immunogold probes specific for *p*-coumaryl-, guaiacyl- and guaiacyl/syringyl-lignins [6], no lignification of the modified primary walls was observed, confirming earlier results obtained with classical histochemical stains [51]. A negative correlation between GRP deposition and lignification was also observed in petunia stems and leaves grown under different light regimes [27].

Both in bean and petunia, growing plant organs were used for the localization of GRPs. In petunia, stems and leaves were examined from plants growing under low light conditions, whereas the work in bean and soybean was mainly conducted with etiolated hypocotyls. The location of GRPs was, however, quite different. Whereas in bean GRP1.8 was located exclusively in the vascular system, ptGRP1 was also located in collenchyma and pith parenchyma. The pattern of expression of *ptgrp1* in young stems was similar to that of transgenic tobacco plants, expressing the *uidA* gene under the control of a bean *grp1.8* promotor containing a deletion that removes a negative regulatory element [62]. This cis-acting element limits gene expression to vascular tissue in transgenic tobacco plants.

Using the immunogold methodology no significant labeling of phloem cells with GRP1.8 antibodies was observed in etiolated bean hypocotyls and in leaves and stem internodes of light-grown bean plants, although a labeling of the protoxylem was consistently observed in these tissues, and unpublished observations [6]. However, using alkaline phosphatase as a detection and amplification system, a clear additional signal was obtained in the region of the phloem of etiolated hypocotyls with tissue prints [5]. In growing petunia stems the phloem was again strongly labeled, using alkaline phosphatase as a detection system. When the reaction product was visualized in the reflection contrast mode with a laser-scanning confocal microscope, the product was localized inside the fluorescent cell wall layer. However, the images did not support a direct association of ptGRP1 with the plasma membrane, the protein being probably located in the secretory system and in the youngest cell wall layers of the labeled petunia cells.

Possible associations of GRPs with other cell wall proteins were studied in etiolated bean and soybean hypo-

cotyls [6]. A polyclonal antibody raised against a 33-kDa proline-rich protein of soybean (PRP2), recognizing the major PRPs of soybean [63]-labeled epitopes in the lignified secondary-wall thickenings of protoxylem and did not react with the modified primary walls, labeled with GRP1.8 antibodies. The secretion of PRPs correlated with the lignification of the secondary walls and preceeded the secretion of GRP1.8 in protoxylem development. In protoxylem and xylem parenchyma cells, Golgi complexes labeled with GRP1.8 antibodies were never labeled with PRP2 antibodies, and vice versa. In conclusion, in protoxylem cells two structural cell wall proteins are deposited in distinct cell wall domains, and are secreted at different time points in protoxylem development. Possibly, interactions of GRP1.8 with other cell wall proteins, or with polysaccharides, remain to be discovered. On the biochemical level, interaction of GRP1.8 with the extracellular matrix was studied by protein extraction experiments. This analysis revealed that GRP1.8 undergoes hydrophobic interactions in the cell wall [26]. Thus, GRP1.8 might be capable of establishing interactions of differents kinds with different components of the extracellular matrix.

Interesting information concerning possible functions of GRP1.8 in the modified walls of protoxylem elements were obtained by the examination of longitudinal sections of etiolated bean and soybean hypocotyls. The walls labeled with GRP1.8 antibodies appeared to be stretched in the longitudinal direction, resembling the stress fibers of fibroblasts in mammals, and were locally attached to the lignified secondary-wall thickenings, as well as to the middle lamella of unlignified xylem parenchyma cells, suggesting that the GRP1.8-containing walls are load-bearing structural elements [6]. GRP1.8 may, therefore, be part of a repair mechanism, reinforcing the hydrolyzed primary walls of the protoxylem, in order to mechanically stabilize the water-conducting elements and to prevent water flow between adjacent protoxylem vessels in extensively growing plant organs (fig. 5). This hypothesis is so far supported by the following observations: (i) the correlation of GRP deposition and extension growth [27, 43]; (ii) the insolubilization of GRP1.8 during protoxylem development in elongating bean hypocotyls [28]; (iii) the hydrophobic interactions established by GRP1.8; (iv) the localization of GUS reaction product in the primary xylem and in ray parenchyma cells, but not in the transition region between the cambium and differentiated secondary xylem of transgenic tobacco plants expressing a full-length *grp1.8* promoter-GUS construct [43, 62]; (v) the stress-fiber-like appearance of the GRP1.8-containing primary walls in bean and soybean, and their apparent attachment to the secondary-wall thickenings of protoxylem elements and to the middle lamella of xylem parenchyma cells; (vi) the staining of the GRP-containing primary walls with Coomassie blue,
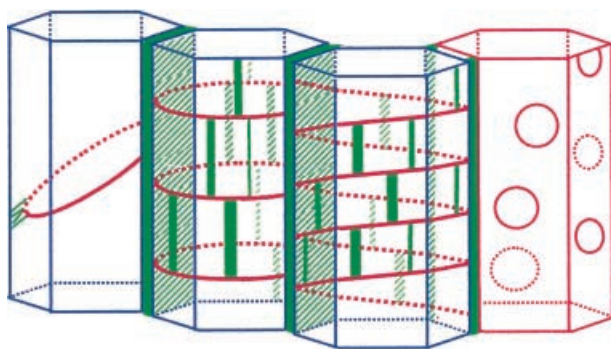
Figure 5. Localization of GRP1.8 in the xylem cells of developing hypocotyls of French bean and soybean. GRP1.8 is marked in green; lignified cell wall thickenings are indicated in red. The youngest protoxylem cells are indicated on the left; metaxylem is on the right. In the longitudinally extending protoxylem, GRP1.8 represents a major component of the modified primary wall, whereas in the nonextensible metaxylem cells it is only found in the cell corners.

Table 3. Aromatic amino acid composition of several well-studied GRPs.

Gene	wGRP1	Osgrp-1	GRP1.8	GRP1
Origin	wheat	rice	bean	petunia
Reference	26	15	14	13
MW (kD)	28.7	13.5	36.5	28.8
aa (mol %)				
Gly	65	56.6	63.1	65.5
Phe	4.6	0.6	0.2	4.6
Tyr	0.0	6.6	6.9	0.0

and (vii) the secretion of GRPs towards the end of protoxylem differentiation by protoxylem cells themselves, as well as by the surrounding xylem parenchyma cells.

Besides the proposed function in a repair mechanism of the elongating protoxylem primary cell walls, Fukuda et al. [64], based on a cell wall GRP found in soybean aleurone layers, propose an additional function for GRPs. The complete primary sequence of this GRP is not known, but the amino terminal sequence of the isolated GRP fraction contains an unusually long stretch of glycine residues [65]. These authors suggest the GRP studied is tightly bound to polysaccharides of cell walls and that it would participate in adhesion between neighboring cells in aleurone layers because of tight binding to the cell wall. Further immunolocalization studies should clarify the location of this GRP and its possible role in cell adhesion in this particular cell layer.

Outlook

Structural proteins in plant cell walls are an underinvestigated field. This is partly due to the difficulty to work with these proteins as they are possibly cross-linked in the wall and are not amenable any more for biochemical studies. In addition, there is no in vitro system for cell wall assembly. The wall is too complex for such a system. Nevertheless, single interactions between purified components of the wall, including structural wall proteins might be studied in vitro. In fact, we have recently studied in vitro cross-linking of fusion proteins between glutathione-S-transferase and two cell wall GRPs: bean GRP1.8 (containing tyrosine) and wheat GRP1 (containing phenylalanine, but no tyrosine) ([26], table 3). Incubating the purified proteins with a peroxidase resulted in

dramatically different results: whereas the GRP1.8 fusion proteins were completely converted to high molecular weight aggregates, the phenylalanine-containing wheat GRP1 was not affected by peroxidase incubation. This strongly implicates the tyrosine residues in peroxidase-mediated crosslinking (intra- or intermolecular) in the wall. Such in vitro systems might clarify which types of interactions occur between the different components of the wall and the GRPs.

The lack of direct functional evidence for GRP action is a major obstacle for a better understanding of the contribution of this class of structural proteins to the living cell wall. Until now, there is no genetic evidence indicating a specific function for GRPs. No mutants in any plant *grp* gene have been described yet. As all plants investigated contain families of *grp* genes, it is likely that the function of the genes shows redundancy, and any mutation in only one *grp* would not result in a phenotype. With the rapid progress of the *Arabidopsis* sequencing project, we will soon know the complete sequence of a plant genome, including a complete set of *grp* genes. The structural cell wall GRPs can then be mutated one after the other by transposon or transfer DNA (T-DNA) mutagenesis to create double or multiple mutants lacking several or all GRPs specifically present in one cell type (e.g., xylem). Such genetic studies should reveal the combined function of GRPs in higher plants. In addition, in vitro and in vivo studies using fusion proteins of GRP domains with reporter proteins will allow study of the interactions of GRP molecules with other cell wall components as well as between themselves. These approaches, combined with further immunolocalization of additional GRPs, should ultimately result in a better understanding of the biological role of these unusual structural proteins of the plant cell wall.

Note added in proof. An excellent review on plant glycine-rich proteins has recently appeared: Sacchetto-Martins G., Franco L. O. and De Oliveira D. E. 2000. Plant glycine-rich proteins: a family or just proteins with a common motif? Biochem. Biophysic. Acta 1492, 1–14.

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