Hydrophobic Interactions of the Structural Protein
GRP1.8 in the Cell Wall of Protoxylem Elements

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The glycine-rich structural protein GRP1.8 of French bean (*Phaseolus vulgaris*) is specifically localized in the modified primary cell walls of protoxylem elements. Continuous deposition of GRP1.8 into the cell walls during elongation growth of the plant suggests that GRP1.8 is part of a repair mechanism to strengthen the protoxylem. In this work, a reporter-protein system was developed to study the interaction of GRP1.8 with the extracellular matrix. Fusion proteins of a highly soluble chitinase with different domains of GRP1.8 were expressed in the vascular tissue of tobacco (*Nicotiana tabacum*), and the chemical nature of the interaction of these fusion proteins in the cell wall compartment was analyzed. In contrast with chitinase that required only low-salt conditions for complete extraction, the different chitinase/GRP1.8 fusion proteins were completely extracted only by a nonionic or ionic detergent, indicating hydrophobic interactions of GRP1.8. The same chemical nature of the interaction of these fusion proteins in the cell wall compartment was analyzed. In contrast with chitinase that required only low-salt conditions for complete extraction, the different chitinase/GRP1.8 fusion proteins were extracted only by a nonionic or ionic detergent, indicating hydrophobic interactions of GRP1.8. The same interactions were found with the endogenous GRP1.8 in bean hypocotyls. In addition, in vitro experiments indicate that oxidative cross-linking of tyrosines might account for the insolubilization of GRP1.8 observed in later stages of protoxylem development. Our data suggest that GRP1.8 forms a hydrophobic protein-layer in the cell wall of protoxylem vessels.

Cell walls are complex structures that confer shape to the cells and ultimately to the whole plant (Varner and Lin, 1989). The primary cell wall of dicotyledonous plants is composed of cellulose microfibrils that are interconnected by hemicelluloses, mainly xyloglucan. This network is the tension-bearing structure of the primary cell wall and is embedded in a matrix of pectins (Varner and Lin, 1989; Carpita and Gibeaut, 1993). Walls can be differentiated and adapted to the particular function of the cell. Xylem vessels are important for water transport and have specialized secondary cell walls that are lignified and thus strengthen and stabilize the elements. The differentiation of xylem involves several steps, e.g. cell elongation, development of lignified secondary cell walls, degradation of the end walls to form a continuous vessel, and programmed cell death, which includes autolysis of the vacuole, plasma membrane, and cytoplasm (Esau and Charvat, 1978; O’Brien, 1981; Fukuda, 1994; Jones and Dangl, 1996; McCann, 1997). The protoxylem is the first xylem developed and has to remain functional during elongation growth of the plant. This requires specific differentiation of the cell wall: (a) Lignin depositions form annular and helical structures that allow passive stretching of the vessels after cell death, and (b) the load-bearing hemicelluloses of the primary cell wall are hydrolyzed to facilitate the passive stretching of the vessels. The modified primary wall consists of coarse fibrils of cellulose, high amounts of proteins, and possibly other compounds of unknown nature (O’Brien, 1981; Ryser et al., 1997).

Three major classes of structural cell wall proteins have been described that are probably important for the mechano-chemical properties of the extracellular matrix: HRGPs (hydroxy-Pro-rich glycoproteins), including extensins and arabinogalactan proteins, PRPs (Pro-rich proteins), and GRPs (Gly-rich proteins) (Keller, 1993; Showalter, 1993; Cassab, 1998). HRGPs and PRPs are insolubilized in tissue under tensile stress or in cells adjacent to a wounding site to strengthen the cell wall (Bradley et al., 1992; Brisson et al., 1994; Tiré et al., 1994; Shirsat et al., 1996). This insolubilization is thought to be caused by oxidative cross-linking of Tyr residues involving H$_2$O$_2$ and peroxidase (Fry, 1982; Waffenschmidt et al., 1993; Schnabelrauch et al., 1996). In the case of HRGPs, Epstein and Lamport (1984) found that Tyr-residues form intramolecular isodityrosine-linkages that are thought to cause the insolubilization of the proteins in the cell wall. A more detailed analysis of a TLKP (Tyr-, Leu-rich protein) showed that a domain of 35 amino acids, rich in tyrosines and cysteines, is sufficient to cross-link a reporter protein in tobacco (*Nicotiana tabacum*) cell walls (Domingo et al., 1999). Although tyrosines are known to be involved in the process of insolubilization, it is not yet clear whether this is taking place uniquely through the formation of intramolecular cross-links or whether also intermolecular linkages between different peptides or between peptides and non-proteinaceous molecules can be formed.

GRP1.8 are characterized by (G-X)$_n$ repeats as the predominant amino acid sequence motif (Condit and Meagher, 1986; Keller et al., 1988; Lei and Wu, 1991).
GRP1.8 of bean (Phaseolus vulgaris) is synthesized by the xylem parenchyma and transported into the modified cell wall of the protoxylem elements (Keller et al., 1988; Ryser and Keller, 1992). Although GRP1.8 is soluble in young tissue, i.e. not fully developed protoxylem, it can no longer be extracted from older protoxylem tissue (Keller et al., 1989b), indicating changes of interactions in the cell wall. Ryser and coworkers (1997) suggested a function of GRP1.8 in a repair mechanism of protoxylem elements. GRP1.8 is present in considerable amounts in the cell wall and might confer specific mechanical properties to the wall that allow the maintenance of the vessels despite ongoing passive elongation during plant growth.

In this study, we have analyzed in more detail the interaction of GRP1.8 with the extracellular matrix. To this end, a reporter-protein system was developed. An extracellular, highly soluble chitinase of cucumber (Boller and Métraux, 1988) was fused to different domains of GRP1.8, and these fusion proteins were expressed in the vascular tissue of transgenic tobacco. Extraction experiments using tissue of plants expressing the chitinase or the chitinase/GRP1.8 fusion proteins, respectively, demonstrate a hydrophobic interaction of GRP1.8 with itself or other components present in the extracellular matrix. These results were confirmed by the analysis of the endogenous GRP1.8 in etiolated bean hypocotyls. A model can be proposed in which GRP1.8 forms a hydrophobic protein-layer that prevents water loss through diffusion across the cell walls of the elongating protoxylem vessels.

RESULTS
Expression of Chitinase and Chitinase/GRP1.8 Fusion Proteins in Transgenic Tobacco

Sequences encoding different domains of GRP1.8 were fused to the 3′ end of a cDNA encoding the cucumber chitinase CUC (Neuhaus et al., 1991). Sequences encoding the N terminus, the middle domain consisting of an almost perfect hexameric repeat of 22 amino acids, and the C terminus of GRP1.8 (Keller et al., 1988; Fig. 1A) were fused to CUC (Fig. 1B), resulting in the fusion constructs CUC, CUC-N, CUC-6R, and CUC-C, respectively. As previous attempts to transform a full-length GRP1.8 gene into tobacco were not successful, such a CUC/GRP1.8 fusion was not constructed. These constructs were expressed in transgenic tobacco under the control of a GRP1.8 promoter that has previously been shown to confer vascular specific gene expression in tobacco (Keller et al., 1989a; Keller and Baumgartner, 1991).

Stem material of 2- to 4-week-old plants grown under sterile conditions was extracted with 1% (w/v) SDS and the extracts were analyzed by western blotting using anti-GRP1.8 and anti-chitinase antiserum (Keller et al., 1988; Me¯traux et al., 1989). In an extract of plants expressing CUC, the anti-chitinase antiserum detected a protein of 28 kD, the expected size of CUC, whereas the extract of non-transgenic plants failed to reveal this band (Fig. 1C). The anti-GRP1.8 antiserum detected several proteins of 60 to 70 kD in total extracts of tobacco (Fig. 1D). In plants expressing CUC-N, CUC-6R, or CUC-C one additional protein was detected corresponding to the transgene-encoded fusion protein (Fig. 1D). Thus, the recombinant proteins were expressed and clearly identifiable in western-blot experiments.

The GRP1.8 Domains Interact in the Cell Wall in a Hydrophobic Manner

In a first step, the complete extraction of the chitinase CUC from the cell wall matrix under low-salt conditions (50 mM sodium-citrate, pH 5.5, subse-
quently referred to as NaC) was established. Ground stem material of tobacco expressing CUC was extracted with NaC, centrifuged, and an aliquot of the supernatant was tested for the presence of the chitinase by western blotting (Fig. 2, left lane). The pelleted crude cell wall fraction was washed extensively with NaC, and an aliquot of the last washing was used to check for complete removal of the NaC-soluble chitinase from the cell wall fraction (Fig. 2, middle lane). Finally, the cell wall fraction was extracted with NaC, 1% (w/v) SDS (referred to as NaC-SDS) to remove remaining soluble proteins, and an aliquot was used to check for the presence of chitinase in this fraction (Fig. 2, right lane). No chitinase was detectable in this last fraction, establishing that soluble chitinase was completely removed from the cell wall fraction by NaC. The same experiment with material of plants expressing the fusion proteins CUC-N, CUC-6R, and CUC-C, respectively, did reveal a consistent extraction pattern that was different from the chitinase alone. Whereas some of the fusion protein was extracted by NaC (Fig. 2, left lane), after extensive washing with NaC, an additional fraction of fusion protein was extracted by the final washing with NaC-SDS (Fig. 2, right lane). Thus, the fusion proteins showed increased interaction in the extracellular matrix compared with CUC alone, which can be attributed to the GRP1.8 protein sequences.

In a next step, a similar extraction experiment was performed with buffers other than NaC-SDS for the final extraction of the cell wall fraction. After a first extraction and subsequent extensive washing with NaC, the plant material was either extracted with NaC, 1% (v/v) Triton X-100 (referred to as NaC-T), a non-ionic detergent, or with the ionic solution 0.5 M CaCl$_2$ (referred to as CaCl$_2$). The results of these experiments done with stem material of a plant expressing CUC-6R are shown in Figure 3. After extraction (Fig. 3, A and B; left lanes) and washing with NaC (Fig. 3, A and B; middle lanes), additional protein was solubilized with NaC-T (Fig. 3A; right lane), whereas no protein was extracted with CaCl$_2$ (Fig. 3B; right lane). The additional extraction of CUC-6R with NaC-T was also found when Triton X-100 was replaced by Nonidet P-40, another hydrophobic compound (result not shown), indicating that a hydrophobic solution is sufficient for the extraction of additional fusion protein. The same experiments with material of plants expressing the fusion proteins CUC-N and CUC-C lead to identical results (data not shown). To confirm these results, experiments were done using NaC-T (Fig. 3C) or CaCl$_2$ (Fig. 3D) for extensive washing of the cell wall fraction, followed by a final extraction with NaC-SDS. No protein was detectable in the NaC-SDS extract after several extractions with NaC-T, indicating that the fusion protein had been completely removed by washing with NaC-T. In contrast, even after extensive washing with CaCl$_2$, extraction of CUC-6R was still possible with NaC-SDS. Again, experiments using plant material of lines expressing CUC-N or CUC-C lead to the same results (data not shown). These findings suggest that each of the different GRP1.8 domains is able to establish hydrophobic interactions within the cell wall matrix and that ionic solutions do not allow the complete extraction of the fusion proteins.

Figure 2. The chitinase/GRP1.8 fusion proteins show interaction with the cell wall in transgenic tobacco. Two hundred milligrams of plant material was extracted with NaC (left lane), washed five times with NaC, and an aliquot of the last washing was loaded to check removal of all protein soluble under these conditions (middle lane). A final extraction was done with NaC-SDS (right lane). Protein detection was performed by western blotting, using anti-CUC and anti-GRP1.8 antisera for CUC and CUC-N, -GR, and -C, respectively. NaC, 50 mM Sodium-citrate, pH 5.5; NaC-SDS, NaC with 1% (w/v) SDS.

Figure 3. Hydrophobic interaction of CUC-6R in the cell wall matrix. Two hundred milligrams of tissue was extracted with NaC (left lane), washed five times under the conditions indicated on top of the middle lanes, and an aliquot of the last wash was used to check removal of all protein soluble under these conditions (middle lane). A final extraction was done as indicated on top of the lanes (right lanes). Protein detection was performed with anti-GRP1.8 antiserum. NaC, 50 mM Sodium-citrate, pH 5.5; NaC-SDS, NaC with 1% (w/v) SDS; NaC-T, NaC with 1% (v/v) Triton X-100; CaCl$_2$, 0.5 M CaCl$_2$. 
The Hydrophobic Interaction of the Fusion Proteins Takes Place in the Cell Wall and Is Quantitatively Significant

An experiment was designed to confirm that the observed hydrophobic interaction of the fusion proteins indeed occurs in the cell wall matrix. An identical experiment as shown in Figure 2 was performed with the modification of an additional centrifugation of the plant extract through a layer of NaC, 40% (w/v) Suc after the first extraction with NaC. After centrifugation, the cell wall fraction is found in the pellet, whereas the membrane and cytoplasmic fractions remain in the supernatant (Price, 1974). As shown in Figure 4A, CUC-6R was still detectable in a NaC-SDS extract of the pellet, indicating that the hydrophobic interaction of the fusion protein is taking place in the extracellular matrix. Identical results were obtained with the other fusion proteins CUC-N and CUC-C (data not shown).

In a next step, the amount of fusion protein recalculated to extraction with NaC was quantified. Plant material expressing the different fusion proteins was either directly extracted with NaC-SDS (total soluble protein) or the cell wall fraction was extensively washed with NaC and finally extracted with NaC-SDS. An aliquot of the latter NaC-SDS extract and a dilution series of the total soluble protein was used for western blot analysis. The results of several independent experiments indicate that for each of the fusion proteins 20% to 50% of the total protein was only extractable with NaC-SDS (Fig. 4B, and data not shown). This demonstrates that a quantitatively significant amount of fusion protein shows hydrophobic interaction with the cell wall matrix and that it is not an unspecific aggregation of a small portion of the fusion protein to the wall.

Hydrophobic Property of the Endogenous GRP1.8 in Bean

To compare the endogenous bean GRP1.8 with the GRP1.8 expressed as a fusion protein in transgenic tobacco, the extraction properties of GRP1.8 from bean hypocotyls were studied. GRP1.8 is at least partially soluble in early but insolubilized in later stages of protoxylem development (Keller et al., 1989b). Therefore, plant material of the upper (younger) and the lower (older) part of etiolated bean hypocotyl was used for the experiment. Ground plant material was extracted with NaC and centrifuged through NaC, 40% (w/v) Suc to purify the wall material. Aliquots of the supernatants were tested for GRP1.8 protein soluble in NaC (Fig. 5; lanes 1 and 4). The pellets containing the purified cell wall fractions were extensively washed with NaC and aliquots of the last washing step were kept as control for complete removal of all soluble GRP1.8 under these conditions (Fig. 5; lanes 2 and 5). A final extraction with NaC-T subsequently was performed (Fig. 5; lanes 3 and 6). In the first NaC-extract of young hypocotyl (lane 1), the anti-GRP1.8 antiserum detected two proteins of 50 and 45 kD, respectively. Additional 50-kD protein was extracted in the final wash with NaC-T (Fig. 5; lane 3), suggesting that it has hydrophobic properties, in contrast with the 45-kD protein that was not detectable in the NaC-T fraction. The single 50-kD protein present in the purified cell wall fraction (Fig. 5; lane 3) corresponds well in size to a bean cell wall protein detected by the anti-GRP1.8 antiserum and to the in vitro-transcribed and -translated GRP1.8 protein (Keller et al., 1988). Thus, the 50-kD protein is likely to represent GRP1.8. In extracts of older hypocotyls, neither the 45-kD nor the 50-kD protein is present. This is in agreement with earlier results that suggested insolubilization of GRP1.8 during protoxylem development. To provide further evidence that the 50-kD protein is indeed GRP1.8 we took advantage of the recent finding that GRP1.8 is digested by collagenase, a proteinase that specifically degrades the triple-helical region of collagen (Webb, 1992; Ryser et al., 1997). As a consequence of this specificity, other proteins such as bovine serum albumin or glutathione S-transferase (GST) are not degraded by collagen (Ryser et al., 1997). Digestion of the different extracts of younger hypocotyl (Fig. 5; lanes 1 and 3) with collagenase resulted in the loss of protein detectable by the anti-GRP1.8 antiserum (Fig. 6A). To exclude that this digestion is due to an unspecific protease activity in the collagenase preparation, a GST-GRP1.8 fusion protein was digested with the collagenase. As described by Ryser et al. (1997), the N terminus of

Figure 4. Protein extraction of a purified cell wall preparation and quantification of the amount of the fusion protein showing hydrophobic interaction. A, Five hundred milligrams of tissue of a plant expressing CUC-6R was extracted with NaC, and the cell wall fraction was purified by ultracentrifugation. Aliquots of the supernatant containing the membrane and cytoplasmic fraction and of the NaC-SDS extract of the purified cell walls were analyzed by western blotting using anti-GRP1.8 antiserum. B, The cell wall fraction of 500 mg of tissue of a plant expressing CUC-6R was either purified, extensively washed with NaC, and then extracted with NaC-SDS (lane 1) or directly extracted with sodium-SDS (lane 2). Decreasing amounts of the aliquot loaded on lane 2 were loaded on lanes 3 through 5. Western blotting was done using anti-GRP1.8 antisera. NaC-SDS, sodium-citrate, pH 5.5, with 1% (w/v) SDS.
GRP1.8 was fused to GST and the purified GST/N fusion protein was incubated with collagenase. While the GRP1.8 moiety of the fusion protein was degraded by the collagenase, the GST remained stable (Fig. 6B), indicating specific degradation of the GRP1.8-domain by collagenase. The disappearance of the extracted cell wall proteins after collagenase digestion provides further evidence that the extracted proteins are indeed GRP1.8 and a structurally closely related protein.

In Vitro Cross-Linking of GRP1.8

Previous work (Keller et al., 1989b) and results of this study indicate insolubilization of GRP1.8 in the cell wall in later stages of protoxylem development. To investigate whether GRPs such as GRP1.8 can be oxidatively cross-linked in vitro and whether Tyr residues in the protein are involved in this process, two different recombinant GRPs were expressed and purified from *Escherichia coli*. wGRP1, a wheat GRP, and GRP1.8 show the similar overall amino acid sequence (G–X)n but differ in one feature: Whereas GRP1.8 contains Tyr-residues, wGRP1 entirely lacks Tyr but contains Phe instead (Fig. 7A). Truncated versions of both GRPs were expressed and purified as GST fusion proteins, incubated with horseradish peroxidase and H2O2, and analyzed by western blotting using anti-GRP1.8 antiserum, respectively. In the presence of H2O2 and peroxidase, the GST-GRP1.8 fusion protein (GST/GRPR) formed high molecular mass complexes that were resistant to boiling in SDS and migrated at ~200 kD. Decreasing the H2O2 concentration caused a gradual disappearance of these complexes (Fig. 7B). The appearance of these complexes concomitant with a strong decrease of the band representing the native GST/GRPR suggests oxidative cross-linking of the fusion protein. The same experiment with the GST-wGRP1 fusion protein (GST/wGRP1D) did not result in significant amounts of high molecular mass complexes detected by the anti-wGRP1 antiserum nor in the disappearance of the native fusion protein (Fig. 7C). A weak signal detectable around 70 kD would correspond in size to a GST/wGRP1 dimer. As the intensity rather increases with decreasing H2O2 concentration, however, the signal most likely does not indicate a product of oxidative cross-linking. Thus, the oxidative cross-linking is dependent on GRP1.8. The main difference between GRP1.8 and wGRP1 is the presence of Tyr in GRP1.8, whereas wGRP1 lacks this amino acid. Other amino acids possibly involved in the formation of covalent bonds such as Ser or Thr are not present in the GRP1.8 domain used in this experiment. This suggests that Tyr residues in GRP1.8 are involved in the observed cross-linking of GRP1.8 in vitro.

DISCUSSION

Hydrophobic Interaction of GRP1.8 in the Cell Wall Matrix

We have developed a reporter-protein system to study the interaction of the structural Gly-rich protein GRP1.8 in the extracellular matrix of the vascular tissue. A chitinase that shows very little interaction with the extracellular matrix and chitinase/GRP1.8 fusion proteins were expressed in transgenic tobacco. Comparison of the extraction patterns of the chitinase reporter protein alone and the different fusion proteins indicate hydrophobic properties of GRP1.8. Corresponding extraction experiments were performed with endogenous GRP1.8 from bean hypocotyl.
cotyl tissue. Although the development of etiolated bean may not be completely identical to the light-grown tobacco, the results confirmed the findings obtained with the reporter-protein system in tobacco. Domingo et al. (1999) have developed a similar reporter-protein system to determine the interactions of an insoluble TLRP in cell walls of a tobacco cell suspension culture. Expression of a chimeric protein consisting of a cystein-rich domain of TLRP and the pathogenesis-related protein PR1 resulted in the insolubilization of PR1 in the cell walls, indicating that the cystein-rich domain is important for the insolubilization of TLRP in vivo. Thus, reporter-protein systems are a valuable tool to analyze properties of structural proteins in the extracellular matrix.

The hydrophobic property of GRP1.8 reveals a new feature of structural cell wall proteins. Other proteins analyzed have shown different biochemical characteristics. Arabinogalactan proteins are highly soluble proteins due to their high content of carbohydrates that can make up to 90% of the molecular mass. Extensins and PRPs are in general also glycosylated, in contrast to the normally unglycosylated GRPs (Showalter, 1993; Cassab, 1998). Polycationic extensins and PRPs rich in Lys and His most likely interact with wall polyanions such as pectic polysaccharides and possibly proteins (Van Dam et al., 1989; Kleis-San Francisco and Tierney, 1990; Miller and Fry, 1992; Kieliszewski and Lamport, 1994). One GRP has been reported to be extractable from soybean seeds by hot water, indicating ionic interactions (Matsui et al., 1995). The sequence of this protein, however, is not known and it appears to be glycosylated, which is unusual for GRPs. The portion of GRP1.8 detectable in the first NaC extract of bean hypocotyl might represent an intracellular pool of protein to be transported from the xylem parenchyma cells into the cell wall. An alternative explanation is that GRP1.8 can undergo ionic interactions. In fact, GRP1.8 contains some amino acids with hydrophilic residues, which might be sufficient to solubilize some of the protein under low-salt conditions.

The absence of soluble GRP1.8 in extracts of older bean hypocotyl is in agreement with the previous report on the insolubilization of GRP1.8 in later stages of protoxylem development (Keller et al., 1989b). Fry (1982) described the correlation of the formation of isodityrosines and cross-linking of cell wall proteins. Inhibition of isodityrosine formation interfered with the observed cross-linking of proteins. This raised the possibility that intermolecular isodityrosine formation might cause insolubilization of proteins in the cell wall. A more precise analysis on isodityrosines in extensins, however, has revealed only intramolecular linkages (Epstein and Lamport, 1984). Thus, although the formation of intermolecular isodityrosine linkages as a mean of insolubilization of cell wall proteins is an interesting hypothesis, their existence remains to be shown. Our experiments demonstrate that GRP1.8 can be oxidatively cross-linked in vitro and that this process is dependent on tyrosines. Future experiments will be designed to reveal whether in muro GRP1.8 is insolubilized by inter- or intramolecular linkages and whether Tyr residues that are present throughout the whole GRP1.8 are involved in this process.

The computational analysis of the secondary and tertiary structure of GRP1.8 indicates how a hydrophobic surface can be formed by a protein that is not genuinely highly hydrophobic. The repetitive domains of GRP1.8 and other GRPs are likely to form a β-pleated sheet (Condit and Meagher, 1986; Keller et al., 1989a).
al., 1988; Lei and Wu, 1991) as suggested by a protein structure prediction program (Gibrat et al., 1987). Due to the (G–X)n motif present in GRP1.8, the non-Gly, hydrophilic amino acids are present in the second position of the repeats and therefore project in the same direction from the β-sheet. Hence, this structure would generate a hydrophobic and a hydrophilic surface and thus a protein with different chemical properties on either side of the β-pleated sheet (Condit and Meagher, 1986; Keller et al., 1988). The uniformity of the amino acid sequence of GRP1.8 with little variation throughout the protein explains the comparable results obtained in the extraction experiments with tobacco expressing the different chitinase/GRP1.8 fusion proteins. The higher order repeat present in the middle domain of GRP1.8 might be important for the correct three-dimensional structure of the protein necessary to execute a particular function. It is well possible that this additional function of GRP1.8 was not revealed by the experimental strategy used in this study.

Function of GRP1.8 in Protoxylem Development

Hydrolysis of the load-bearing hemicelluloses of the primary wall is an integral part of protoxylem development. Intact and functional primary walls, however, are required for functioning of the protoxylem, and thus, some component(s) of the primary cell wall must functionally replace the hydrolyzed hemicelluloses. In addition, weakening of the cell walls as a consequence of continuous elongation requires an effective repair mechanism to prevent collapsing of the vessels and water loss through diffusion into the surrounding tissue. The deposition of considerable amounts of hydrophobic GRP1.8 during protoxylem development suggests a role in preventing water loss through diffusion and strengthening of the cell wall by insolubilization of the protein.

At this point, it is unclear whether GRP1.8 forms an independent network or whether it closely interacts with other components of the cell wall. Methylated pectins and lignin are both hydrophobic compounds (Brett and Waldron, 1996) and therefore could establish hydrophobic, possibly also covalent bonds with GRP1.8. In fact, GRP1.8 spans the region between the lignified ring structures of protoxylem elements and might physically interconnect them (Ryser et al., 1997). The localization of GRP1.8 to non-lignified parts and the absence of detectable GRP1.8 in the cell wall of continuously lignified secondary xylem raise the question whether GRP1.8 functionally replaces lignin. The hydrophobic property and the proposed function in strengthening of the cell wall for both lignin and GRP1.8 are in agreement with this hypothesis. The different interactions of GRP1.8 might also reflect a change in the function of GRP1.8 during the progression of protoxylem development. While in early stages, prevention of water loss by the hydrophobic property could be the main function of the protein, its wall-enforcing covalent interactions might become more important later in development. Additional ionic and/or covalent interactions of GRP1.8 with lignin or polysaccharides are possible. Several speculative suggestions of polysaccharide-protein cross-links through a hydroxycinnamic-acid linker have been made but remain to be proven (Iiyama et al., 1994 and references therein). In these models, Cys and Tyr residues would be involved in the interactions. The insolubilization of GRP1.8 may be caused by oxidative cross-linking between individual GRP1.8 proteins, as shown in the in vitro experiments, and/or between GRP1.8 and cellulose microfibrils. This would result in a GRP1.8-cellulose network that reinforces the cell wall. Such a mechanism has been reported for extensin, which can increase the strength of the tissue by the formation of an extensin-cellulose framework (Iraki et al., 1989) and thus functionally replace xyloglucan (Carpita and Gibeaut, 1993). GRPs found in spider silk are thought to provide elasticity and stability (Lewis, 1992) and GRP1.8 could ensure that protoxylem remains stable and flexible despite the ongoing elongation process.

The hypothesis of a GRP1.8-cellulose network, however, remains speculative. There is little information on other components and putative interaction partners of GRP1.8 present in the cell wall. Despite the lack of conclusive knowledge on the exact function of GRP1.8, our data support the model that GRP1.8 is deposited in the modified primary cell wall of protoxylem elements as part of a repair mechanism to maintain the functionality of the vessels during continuous longitudinal growth of the plant. Further analysis of the biochemical properties of GRP1.8 and the study of grp knock-out mutants in other plant systems such as Arabidopsis should allow the more precise analysis of the function of GRPs in the process of protoxylem development. Also, it will be interesting to test which domains and individual amino acids of GRP1.8 are involved in the covalent cross-linking of the protein in the extracellular matrix and whether the hydrophobic property found for GRP1.8 is a characteristic that holds true for Gly-rich structural proteins in general.

MATERIALS AND METHODS

Plasmids and Gene Constructs

The vector pSCU1 encoding the cucumber chitinase was described by Neuhaus et al. (1991). A KpnI and a BglII site were introduced in front of the stop codon of CUC. To this end, two PCR products were obtained using the primers Chiz/Chi2 and Chiz/Chi4 and pSCU1 as template, digested with NsiI and XbaI, respectively, and used for triple ligation with pSCU1, cut with NsiI/XbaI. The N and C terminus of GRP1.8 (amino acids 27–200 and 370–458, respectively) (Keller et al., 1988) were amplified by PCR.
using the primers grp1/grp2 and grp3/grp4, respectively, digested with KpnI/BglII and cloned into pSCU1 cut with the same enzymes. The domain 6R was cloned by ligating a GRP1.8 deletion encoding amino acids 186 to 333 into pSCU1 cut with KpnI/BglII.

The construction of the GST/N fusion was described by Ryser et al. (1997). GST/GRPR was obtained by cloning a GRP1.8 deletion encoding amino acids 203 to 333 into pGEX-4T-3 cut with EcoRI/XhoI. The CUC and CUC/GRP1.8 fusion constructs under the control of the GRP1.8 promoter were cloned by a triple ligation of the individual CUC and CUC/GRP1.8 constructs digested with BamHI/EcoRI, a 514-bp GRP1.8 promoter fragment cloned by PCR using the primers GRPPROM5/ GRP6 and digested with BamHI/XhoI, and the plant transformation vector pBI101 digested with SalI/EcoRI, resulting in pBIpSCU1/-N/-6R/-C.

DNA Oligonucleotides Used for PCR or as Linker Sequences
The following oligonucleotides were used in this work: Chi1 5’ CCGCCCATGC 3’, Chi2 5’ CCGGTACCCTTCTCAC 3’, Chi4 5’ TCGACTCTAG 3’, GRP1 5’ CCGCCAATTC 3’, GRP2 5’ GGGAGATCTCCATACCCTC 3’, GRP3 5’ CCGGTACCGATGCTTAATGCTAGCAGTC 3’, GRP4 5’ CCGGTACCCTTCTCAC 3’, GRP5 5’ CCGGTACCGATGCTTAATGCTAGCAGTC 3’, GRP6 5’ CCGGAATCTC- CGGTTTTGAAGTGAGG 3’.

Cloning of wGRP1 and Generation of Anti-wGRP1 Antiserum
To isolate wGRP1, 9 × 10⁸ phages from a genomic EMBL3-library of Triticum aestivum var Cheyenne were screened with the GRP1.8 coding region as a probe. The DNA of two positive clones was isolated and sequenced and both encoded wGRP1 (EMBL data bank, accession no. AJ276509). For overexpression of wGRP1, the sequence had to be adjusted to the Escherichia coli codon usage. To this end, four doublestranded-oligonucleotides encoding the amino acids 256–289, 290–325, 326–359, and 360–390 of wGRP1, respectively, were ligated together and cloned into pGEX-4T-2 opened with EcoRI/XhoI, resulting in the sequence encoding GST/wGRP1D (EMBL data bank, accession no. AJ276243). Anti-wGRP1 antiserum was obtained by immunization of rabbits with purified GST/wGRP1D fusion protein. A purified IgG fraction of the antiserum was used for western blotting. It was confirmed that the preimmune serum did not recognize the GST/wGRP1D fusion protein.

Plant Transformation and Growth
Tobacco (Nicotiana tabacum) plants were transformed by the Agrobacterium tumefaciens-mediated leaf disc transformation method as described by Keller and Baumgartner (1991). Transgenic tobacco plants were propagated in jars with 3% (w/v) Suc, 1× Murashige and Skoog medium, 1% (w/v) agar, and grown in a climate chamber (16-h photoperiod, 25°C).

French bean (Phaseolus vulgaris L.) seeds were put into soil, watered, and kept in the dark for 10 d for etiolation.

Plant Protein Extraction Procedures
Transgenic tobacco plants were grown under sterile conditions to a height of 7 cm and the stem material was ground in liquid N₂ in a mortar to a fine powder and stored at −80°C. The hypocotyls of the soil-grown etiolated bean plants were cut into three parts of equal length, and the upper and lower thirds (corresponding to younger and older tissue, respectively) of five plants were separately pooled, ground in liquid N₂ in a mortar to a fine powder, and stored at −80°C.

Except when using SDS, all work was done on ice. For extractions used for western blotting, plant material (200 mg) was extracted with 200 μL of appropriate buffer by vortexing, centrifuged for 10 min at 13,000g at 4°C, and 40 μL of the supernatant was used for analysis. For extensive washing, the pellet was washed 5× with 1.5 mL of appropriate buffer by vortexing and centrifuged as indicated above. To verify removal of all protein soluble in washing buffer, an extraction was done with 200 μL, and 40 μL was used for western blotting.

For the purification of the cell wall extract, 500 mg of ground plant material was extracted with 600 μL of NaCl, and the suspension overlayed on 10 mL NaCl, 40% (w/v) Suc for ultracentrifugation (100,000g, 4°C for 1 h) (Beckman, SW41Ti rotor). The supernatant, containing the membrane and the cytosolic fraction, was kept on ice, the NaCl (40% [v/v] Suc solution) was decanted, and the pellet containing the purified cell wall fraction was washed and extracted as described above. Forty microliters per 600-μL fraction was used for western blotting.

Expression and in Vitro Cross-Linking of GST/GRP Fusion Proteins
The expression and purification of the different GST-fusion proteins was performed according to a published protocol (Ryser et al., 1997). The purified fusion proteins were eluted from a Fast Desalting column in 50 mm NaHCO₃, pH 9.0. Two micrograms of protein was incubated with 0.275 unit of horseradish peroxidase, type XII (Sigma, Buchs, Switzerland) and H₂O₂ as indicated at 25°C for 15 min. The reaction was stopped by heat denaturation in SDS sample buffer for 3 min at 95°C, and the samples analyzed by western blotting.

Collagenase Digestion
The protein extracts were dialyzed overnight at 4°C against 20 mm Tris, 3 mm CaCl₂, pH 7.5. Eight micrograms of plant protein fraction and 600 ng of GST/N fusion protein were incubated with 4 units of collagenase (Sigma) for 3 h at 40°C. The reactions were stopped by heat dena-
turation in SDS-PAGE loading buffer and analyzed by Coomassie staining for the GST/N fusion protein and by western blotting for the plant protein extracts.

SDS-PAGE and Western Blotting

Proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membrane by the semidy method using the Transblot SD Semidy Transfer Cell (Bio-Rad Laboratories, Glattbrugg, Switzerland). After blocking overnight in 1× Tris-buffered saline plus Tween 20 (TBST) and an additional hour in 1× TBST, 2.5% (w/v) bovine serum albumin, 5% (w/v) low fat milk powder, antibody incubation, and washing was performed with 1× TBST, 0.5% (w/v) low-fat milk powder. Primary and secondary antibodies were diluted 1:4,000. For detection, the enhanced chemiluminescence kit and Hyperfilm-ECL (Amersham, Dübendorf, Switzerland) were used following the manufacturers’ instructions.

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