

IMMUNOHISTOLOGICAL STUDY OF MANNAN POLYSACCHARIDES IN POPLAR STEM

HSIN-TZU WANG, I-HSUAN LIU** and TING-FENG YEH*

*School of Forestry and Resource Conservation, National Taiwan University,
Taipei 10617, Taiwan

**Department of Animal Science and Technology, National Taiwan University,
Taipei 10617, Taiwan

Received November 21, 2011

Mannan polysaccharides serve as storage reserves in seeds and as structure elements in cell walls, but they may also perform other important functions during plant growth. As one of the major hemicelluloses in angiosperm wood, little is known about the presence and localization of mannan polysaccharides during xylem development in the model tree, *Populus trichocarpa*. In this study, we used mannan-specific recognized antibody to label mannan polysaccharides in stem tissues at different developmental stages. Immunofluorescence microscopy showed that the epitopes were localized in xylem elements, especially in thickened secondary cell walls and interfascicular fibers, while other cell types revealed a low level of mannan epitopes. The signals were possibly masked by acetylation, glucuronoxylans or pectic polymers depended on cell types, but were less affected by lignification. These results demonstrate that mannans are of particular significance in the secondary cell walls of the xylem tissue, which provides us a further opportunity to study the biosynthesis of mannans during xylem development in wood.

Keywords: carbohydrate-binding module (CBM), immunofluorescence, mannan polysaccharides, monoclonal antibody, plant cell walls

INTRODUCTION

Plant cell walls not only play a key role in cell differentiation, intercellular communication and cell defense, but also provide rigidity to plant body, while giving flexibility during cell expansion. The cell walls are highly organized composites, which contain a variety of polysaccharides that are grouped into cellulose, hemicelluloses and pectic polysaccharides.¹ Cellulose forms a load-bearing microfibril network cross-linked by hemicelluloses embedded in a matrix of pectin. The relative abundance of these hemicellulose polysaccharides varies between primary and secondary cell walls, depending on cell type and developmental status.

Polysaccharides containing β -1,4-linked mannosyl residues, referred to as mannan polysa-

ccharides, are widespread and found as cell wall components in angiosperms and gymnosperms,² such as mannans, glucomannans, galactomannans and galactoglucomannans. Mannans serve as structural polymers, taking the place of cellulose as the primary structural polysaccharide in some algal species,³ and are found in the thickened endosperm walls as storage polymers of palm seeds (e.g. ivory nut [*Phytelephas macrocarpa*]).⁴ Glucomannans consist of β -1,4-linked mannosyl residues interspersed by β -1,4-linked glucose; they are present in plant secondary cell walls and have been observed in vegetative tissues of a variety of monocot species.⁵ Glucomannans may also influence the progression of embryogenesis in *Arabidopsis*.⁶ Galactosyl residues are

α -1,6-linked to mannan backbones in galactomannans, these polymers being particularly abundant in cell walls and vacuoles of endosperm cells in legume seeds as storage polymers.⁷ Galactoglucomannans have glucomannan backbones substituted with α -1,6-linked galactosyl residues, they are abundant in the thickened secondary walls of gymnosperms.^{8,9} They have also been shown to influence the differentiation of *Zinnia* tracheary elements *in vitro*, which reveals that galactoglucomannans may function as signaling molecules in development.¹⁰

Mannan polysaccharides are functionally diverse, their amounts and structures vary greatly between the secondary cell walls of softwoods and those of hardwoods. *O*-acetyl-galactoglucomannans are the principal hemicelluloses in softwoods (about 20%) with a degree of polymerization (DP) of 100, and the ratio of glucosyl to mannosyl residues can vary from 1:4 to 1:3. The minor hemicellulose in softwood is 4-*O*-methyl-arabinoglucuronoxylan (5-10%). However, hardwoods contain only 2-5% of glucomannans with a DP of 200, and the ratio of glucosyl to mannosyl residues varies between 1:2 and 1:1, the major hemicellulose in hardwood being actually *O*-acetyl-4-*O*-methyl-glucuronoxylan (15-30%).¹¹ Despite the importance of mannan polysaccharides to the plant, the presence of mannan polysaccharides in plant cell walls during xylem development has not yet been clearly demonstrated, and the contributions of mannan polysaccharides to the properties displayed by cell walls are far from clear. In this study, a mannan-specific monoclonal antibody, LM21,¹² was used to detect mannan polysaccharides in a model woody plant, *Populus trichocarpa*, during wood formation. The results provided evidence that the accumulation of mannan polysaccharides was consistent with xylem development, which demonstrated that the regulated quantities of mannan polysaccharides were important during xylogenesis.

MATERIALS AND METHOD

Plant material and growth conditions

Wild-type black cottonwood (*Populus trichocarpa* Torr. & A. Gray) was received from Dr. Vincent L.

Chiang, North Carolina State University. It was planted in peat:perlite:vermiculite (1:1:1) with fertilizer and grown at 25/20 °C (day/night), in a greenhouse, under a regime of 16 h of light and 8 h of dark, with natural lighting supplemented with artificial lamps. The plants were watered daily, without supply of other nutrients.

Sample preparation for microscopy

Stem segments of different internodes were collected from 4-month-old plants and fixed in formalin-acetic acid-alcohol (FAA) overnight at 4 °C. After fixation, the tissues were dehydrated in an ethanol series consisting of 50, 60, 70, 85 and 95% ethanol (1 h each), and embedded in wax. Transverse sections were cut (10 μ m) with a rotatory microtome and transferred to glass slides for immunofluorescence microscopy. Micrographs of different histological staining groups were taken and compared within the same internode, leaving about 6 cuts (60 μ m) between the staining groups compared.

Sample pretreatment and lignin staining before microscopy

Sections on glass slides were dewaxed and pretreated under different conditions for comparison. The pretreatments included 1 M potassium hydroxide at 25 °C for 1 h¹² or 8% NaClO₂ in 1.5% acetic acid at 40 °C with incubation time of 1 h. Freshly prepared phloroglucinol/hydrochloric acid solution was used for lignin color staining, according to Nakano and Meshitsuka.¹⁴

Probes

Rat mannan-directed monoclonal antibody, LM21, binds most effectively to β -1,4-manno-oligomers with DP from 2 to 5, glucomannan and galactomannan polysaccharides, as described previously.¹² A carbohydrate-binding module (CBM), CBM3a, specific to crystalline cellulose,¹⁵ was used as a counter-labeling for visualization of all cell walls. Both of the probes were purchased from Plant Probes (Leeds, UK).

Immunofluorescence microscopy

Transverse sections were blocked with 3% (w/v) bovine serum albumin (BSA) in phosphate-buffered saline (PBS; 137 mM sodium chloride, 2.7 mM potassium chloride, 10 mM sodium phosphate, 2 mM potassium phosphate, pH 7.4) (BSA/PBS) for 30 min at room temperature to reduce non-specific labeling. Sections were then incubated with a 5-fold dilution of monoclonal rat antibody, specific to mannan polysaccharide epitopes, in BSA/PBS, for 1 h at room temperature, with gentle shaking. Following washing with PBS at least three times, anti-mouse antibody linked to fluorescein isothiocyanate (anti-mouse FITC;

Sigma) was applied as a 100-fold dilution in BSA/PBS, for 1 h, with gentle shaking in the dark. The samples were washed at least three times again with PBS, mounted in Citifluor anti-fade reagent (Agar Scientific), and observed on a Leica DM2500 microscope equipped with epifluorescence irradiation. Micrographs were obtained using a Canon EOS 7D camera. For His-tagged CBM labeling, a triple indirect immunofluorescence labeling procedure was needed as described in literature.¹⁵ Controls without incubation with primary antibody were also prepared and compared.

RESULTS AND DISCUSSION

Deposition of mannan polysaccharides in *P. trichocarpa* during xylogenesis

The result of immunofluorescence labeling clearly shows that cellulose deposited almost everywhere in different cell types and stages (Fig. 1, CBM3a). However, mannan polysaccharides were restricted to primary xylem in an early developmental stage, and the signals became stronger in secondary xylem during the formation of the secondary cell wall (Fig. 1, LM21). The labeling intensity during xylem development demonstrated that mannan polysaccharides had accumulated during wood formation. This was similar to the association of mannan deposition with the formation and maturation of sclerenchyma cells and xylem elements observed in *Vicia faba*.¹⁶ A comparable finding was also reported in secondary cell wall during tracheid development in *Cryptomeria japonica*.¹⁷

The labeling by both CBM3a and LM21 seemed to have an increased and then decreased intensity in xylem during development. It was assumed that the decreased intensity was caused by the accumulation of lignin, which might obscure the epitopes, as described previously in differentiating tracheids of *Chamaecyparis obtusa* during cell wall formation.¹⁸ To observe the cause of this phenomenon, we pretreated sections with NaClO₂ to remove lignins before immunolabeling. The results of phloroglucinol staining revealed that lignins were largely removed after NaClO₂ treatment (Fig. 2), however, the following LM21 labeling (Fig. 1, NaClO₂/LM21) showed there was no significant difference in epitope recognition, compared to that of sections without NaClO₂ pretreatment. The

intensity and distribution of epitopes seemed not affected by delignification, indicating that lignins may not be the predominant components involved in the masking of LM21 labeling in poplar stem tissues. Kim *et al.*¹³ also reported that there were no significant differences in *O*-acetyl-galactoglucomannan-labeled compression wood tracheid of *Cryptomeria japonica* before and after delignification. These suggested that lignins might not be the main components associated with the decreased intensities of mannan epitopes, and other components such as pectin or glucuronoxylan might play a role in this.

The secondary cell walls in phloem fibers were also labeled, and the epitopes seemed to be much more obvious in the outer layer where phloem fibers formed earlier (Fig. 1, LM21, the 12th internode). This indicated that the accumulation of mannan polysaccharides may have preferential deposition in earlier-formed phloem fibers, or the epitopes were presumably masked by the acetylation of mannan polysaccharides, as reported a widespread phenomenon appeared in intact secondary cell walls.¹² Sections were also treated with alkali (1 M KOH for 1 h) to remove the acetyl group, and followed by LM21 labeling again. The results (Fig. 1, KOH/LM21) showed that the different intensities of epitopes observed in untreated phloem fibers no longer existed, suggesting that LM21 signals were masked by acetylation in secondary cell walls. The results also reflect the intrinsic aspect of acetylation heterogeneity across the secondary cell walls of phloem fibers formed in different developmental stages, and may affect the interaction of hemicellulose polysaccharides in cross-linking with other cell wall components, such as cellulose microfibrils.¹⁹ On the other hand, it is worth noting that LM21 signals in sections (not only in phloem fibers) from the 4th internode were dramatically increased after KOH treatment, especially in the cell walls of xylem and pith parenchyma (Fig. 1, KOH/LM21). Similar observations have been made in LM21 labeling to tobacco stem sections that 1 M KOH treatment greatly increased probe recognition in secondary walls of xylem cells and

phloem fibers.¹²

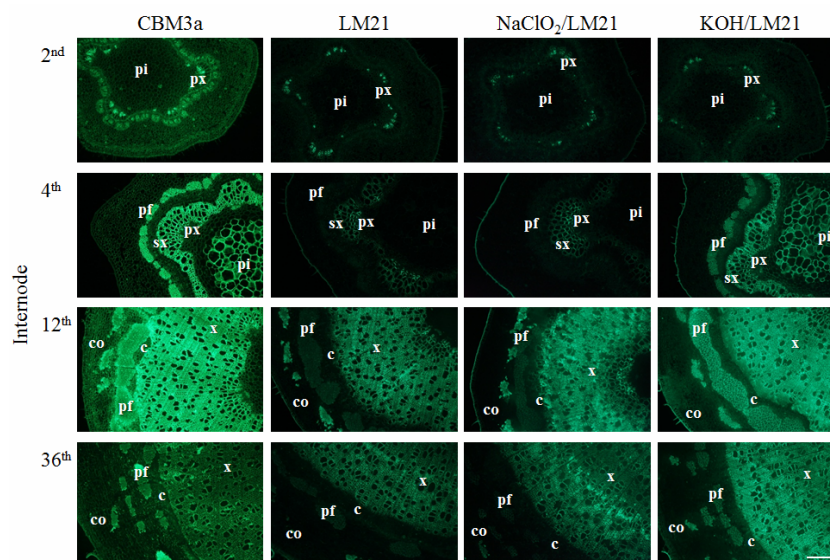


Figure 1: Immunolabeling of mannan polysaccharides in poplar stem tissues with and without NaClO_2 and KOH pretreatments from 2nd, 4th, 12th and 36th internode by using LM21. All cell walls were visualized by labeling of cellulose-directed CBM3a (c – cambium; co – cortex; pf – phloem fiber; pi – pith; px – primary xylem; sx – secondary xylem; x – xylem. Bar = 200 μm)

O-acetyl-4-O-methyl-glucuronoxylan, sometimes referred to as glucuronoxylan (about 15-30% of dry wood weight), was the major hemicelluloses component in hardwood xylem, whereas glucomannan was the minor component in hardwood xylem (about 2-5% of dry wood weight), and usually without acetyl group compared to galactoglucomannan in softwood.¹¹ The masking components removed by 1 M KOH treatment from the secondary wall of xylem cells might not be the acetyl groups attached to mannan polysaccharides. The lower strength of alkali (1 M or *ca.* 5% KOH) has been reported to solubilize small but significant amounts of glucuronoxylans, xylans, xyloglucans, galactoglucomannans and pectic polymers, while most of the glucomannan could only be removed with higher alkali concentrations – of 16 or 24% KOH or 17.5% NaOH.^{20,21} The use of 1 M KOH resulted not only in de-esterification, but also in the removal of sets of polysaccharides (presumably glucuronoxylans in xylem and pectic polymers in pith parenchyma), which could lead to the exposure of hidden epitopes and improve the recognition of probes both in primary and

secondary cell walls. This is a possible explanation for the occurrence of LM21 signals in the cell walls of xylem and pith parenchyma after alkali treatment (Fig. 1, KOH/LM21), which also reveals that mannan polysaccharides are components of hemicellulose polymers in both the cell walls of xylem and pith parenchyma in poplar stem.

The labeling was observed in the cell walls of epidermis, which is an important cell layer controlling plant growth.²² The cell walls in the cortex and cambial zone were not labeled, revealing that there may be little mannan polysaccharides present in these parenchyma systems, or the mannan epitopes are masked by other competitive polysaccharides.¹² Further experiments are necessary to find out the true identity of removed polysaccharides during 1 M KOH pretreatment.

Recognition of mannan-specific epitopes in secondary cell walls

The localization of mannan polysaccharides in xylem was less labeled in the cell walls of ray parenchyma (Fig. 3b), when compared to that of

interfascicular xylem fiber or vessel element. This demonstrates that rays may have a lower amount of mannan polysaccharides or a delayed deposition of mannan polysaccharides in their walls, as shown previously in developing secondary xylem of hybrid poplar (*Populus deltoides* × *P. trichocarpa*).²³ However, in earlywood of *Cryptomeria japonica*, Kim *et al.*²⁴ reported either an earlier or a later deposition of galactoglucomannan in ray cells at an early stage of S₁ formation in tracheids, indicating that mannan deposition in the ray cells from different species might be regulated differentially. A restricted distribution of epitopes was observed in the thickened secondary cell walls of xylem fibers (Fig. 3b). A similar distribution was noted for *Arabidopsis* stem tissues, where anti-mannan antisera showed strong labeling in thickened cell walls of xylary cells and interfascicular fibers.²⁵

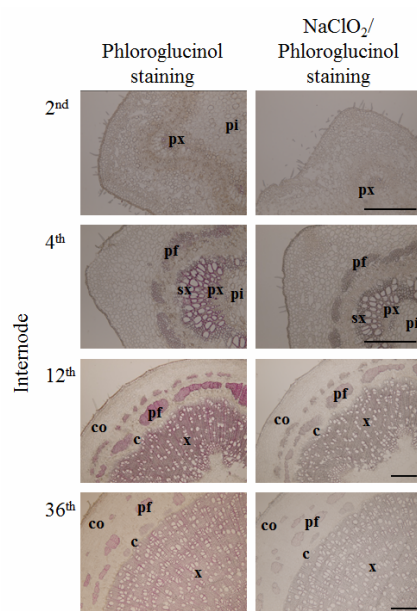


Figure 2: Phloroglucinol staining of lignins in poplar stem sections from different internodes with NaClO₂ pretreatment (c – cambium; co – cortex; pf – phloem fiber; pi – pith; px – primary xylem; sx – secondary xylem; x – xylem. Bar = 200 μm)

Contrary to the phenomena observed in xylem elements, cell junctions and intercellular spaces of

The thickened secondary cell walls of the vessels were also labeled, but no epitopes were noted in the compound middle lamella and cell corners (Fig. 3b), where lignins were first detected after the cells completed the deposition of S₁ layer.^{18,26,27} Again we presumed that lack of epitopes in these sites was probably caused by lignification. Further investigation by comparing with either NaClO₂ or KOH pre-treated samples revealed that LM21 signals did not increase in cell junctions and middle lamella after the current condition of delignification (Fig. 3c) and deacetylation or pectic polymer removal (Fig. 3d). This suggested that the reduced LM21 occurrence in cell junctions and middle lamella of xylem fibers was caused either by other masking polymers or by no mannan deposition rather than by lignin obscuring in this site.

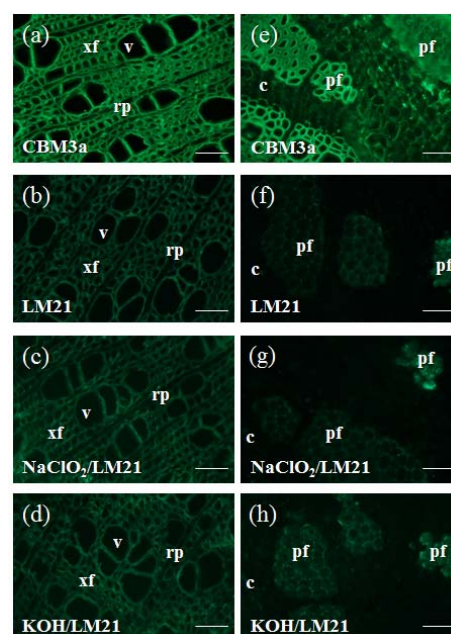


Figure 3: Immunolabeling of mannan polysaccharides in secondary xylem (b-d) and phloem (f-h) of poplar stem tissues using mannan-directed monoclonal antibody, LM21. Cellulose-specific CBM3a was used to visualize all cell walls in xylem (a) and phloem (e) (c – cambium; xf – xylem fiber; pf – phloem fiber; rp – ray parenchyma; v – vessel element. Bar = 50 μm)

phloem fibers were bound by LM21 (Fig. 3f), suggesting that mannan polysaccharides may play

a role in facilitating the adhesion between adjacent phloem fibers, which could possibly improve the physical mechanism of plant body. After alkali treatment (Fig. 3h), LM21 signals were not only restricted to the cell junctions between phloem fibers, but also distributed in sclerified secondary cell walls of phloem fiber, revealing that mannan polysaccharides may serve as structural polymers in the secondary walls of phloem fibers and presumably contribute to strengthening the physical properties of plants as well. The occurrence of cell junctions and cell wall lining intercellular spaces of the primary cell wall for tobacco and pea parenchyma cell walls was also reported previously.¹² The differences of mannan polysaccharide distribution among different cell types provided evidence that the deposition of mannan polysaccharides was well-regulated during different developmental stages of plant growth, and the coexistence in different cell walls and cell wall junctions indicated that they may play a role in supporting structural functions.

CONCLUSIONS

As some aspects of mannan polysaccharide occurrence in plant cell walls have not been elucidated, this study aimed at exploring mannan depositions during the development of woody plants. The results showed that mannan epitopes are present in the primary xylem at the beginning of xylem development, and during plant growth, the labeling intensities increased in secondary xylem, especially in the secondary cell walls of vessels and xylem fibers. The epitopes were also observed in the intercellular spaces and thickened secondary cell walls of phloem fibers, revealing that mannan polysaccharides may serve as structural polymers in cell walls during xylogenesis. The application of monoclonal antibodies and CBMs in investigating polysaccharide distribution in intact cell walls was very informative, and the potential masking of epitopes by other cell wall components should be considered cautiously. In our case, LM21 epitopes were possibly masked by acetylation, glucuronoxylans or pectic polymers depending on cell type, but were less affected by lignification.

The hidden epitopes masked by different cell wall polymers also remind us that the heterogeneous nature of cell wall structures in different developmental stages. The molecular mechanisms regulating mannan polysaccharides synthesis are just beginning to be understood, and more efforts are still needed to explore the roles and functions of mannan polysaccharides involved in cell wall assembly. We hope that the results of this study could provide a further opportunity to study the biosynthesis of mannans during xylem development.

ACKNOWLEDGMENTS: The authors gratefully acknowledge the National Science Council of Taiwan (NSC 98-2313-B-002-063-MY2) for the financial support. Also, the authors are grateful to Dr. Vincent L. Chiang (North Carolina State University) for his valuable suggestions to this manuscript.

REFERENCES

- ¹ M. A. O'Neill and W. S. York, in "The Plant Cell Wall", edited by J. K. C. Rose, Blackwell Publishing, 2003, pp. 1-54.
- ² A. Bacic, P. J. Harris and B. A. Stone, in "The Biochemistry of Plants – A Comprehensive Treatise", edited by J. Preiss, Academic Press, 1988, pp. 297-371.
- ³ W. Mackie and R. D. Preston, *Planta*, **79**, 249 (1968).
- ⁴ M. K. Matheson, *Methods Plant Biochem.*, **12**, 371 (1990).
- ⁵ H. Meier and J. S. G. Reid, in "Encyclopedia of Plant Physiology", edited by F. A. Loewus and W. Tanner, Springer, 1982, pp. 418-471.
- ⁶ F. Goubet, C. J. Barton, J. C. Mortimer, X. Yu, Z. Zhang, G. P. Miles, J. Richens, A. H. Liepman, K. Seffen and P. Dupree, *Plant J.*, **60**, 527 (2009).
- ⁷ M. S. Buckeridge, H. P. dos Santos and M. A. S. Tine, *Plant Physiol. Biochem.*, **38**, 141 (2000).
- ⁸ P. Capek, M. Kubachova, J. Alfoldi, L. Bilisics, D. Liskova and D. Kakoniova, *Carbohydr. Res.*, **329**, 635 (2000).
- ⁹ J. Lundqvist, A. Teleman, L. Junel, G. Zacchi, O. Dahlman, F. Tjerneld and H. Stalbrand, *Carbohydr. Polym.*, **48**, 29 (2002).
- ¹⁰ A. Benova-Kakosova, C. Dignonnet, F. Goubet, P. Ranocha, A. Jauneau, E. Pesquet, O. Barbier, Z. N. Zhang, P. Capek, P. Dupree, D. Liskova and D. Goffner, *Plant Physiol.*, **142**, 696 (2006).
- ¹¹ E. Sjöström, "Wood Chemistry: Fundamentals and Applications", San Diego, Academic Press, 1993, 293 p.
- ¹² S. E. Marcus, A. W. Blake, T. A. S. Benians, K. J. D.

- Lee, C. Poyser, L. Donaldson, O. Leroux, A. Rogowski, H. L. Petersen, A. Boraston, H. J. Gilbert, W. G. T. Willats and J. P. Knox, *Plant J.*, **64**, 191 (2010).
- ¹³ J. S. Kim, T. Awano, A. Yoshinaga and K. Takabe, *Planta*, **233**, 721 (2011).
- ¹⁴ J. Nakano and G. Meshitsuka, in "Methods in Lignin Chemistry", edited by S. Y. Lin and C. W. Dence, Springer-Verlag, 1992, pp. 23-32.
- ¹⁵ A. W. Blake, L. McCartney, J. E. Flint, D. N. Bolam, A. B. Boraston, H. J. Gilbert and J. P. Knox, *J. Biol. Chem.*, **281**, 29321 (2006).
- ¹⁶ W. Chen, F. L. Stoddard and T. C. Baldwin, *Int. J. Plant Sci.*, **167**, 919 (2006).
- ¹⁷ J. S. Kim, T. Awano, A. Yoshinaga and K. Takabe, *Planta*, **232**, 545 (2010).
- ¹⁸ Y. Maeda, T. Awano, K. Takabe and M. Fujita, *Protoplasma*, **213**, 148 (2000).
- ¹⁹ S. E. C. Whitney, J. E. Brigham, A. H. Darke, J. S. G. Reid and M. J. Gidley, *Carbohydr. Res.*, **307**, 299 (1998).
- ²⁰ D. Fengel and G. Wegener, "Wood: Chemistry, Ultrastructure, Reactions", New York, Walter de Gruyter, 1989, 613 p.
- ²¹ R. R. Selvendran and M. A. O'Neill, in "Methods of Biochemical Analysis", edited by D. Glick, John Wiley & Sons, 1987, pp. 25-153.
- ²² S. Savaldi-Goldstein, C. Peto and J. Chory, *Nature*, **446**, 199 (2007).
- ²³ M. Kaneda, K. Rensing and L. Samuels, *J. Integr. Plant Biol.*, **52**, 234 (2010).
- ²⁴ J. S. Kim, T. Awano, A. Yoshinaga and K. Takabe, *Planta*, **233**, 109 (2011).
- ²⁵ M. G. Handford, T. C. Baldwin, F. Goubet, T. A. Prime, J. Miles, X. L. Yu and P. Dupree, *Planta*, **218**, 27 (2003).
- ²⁶ I. W. Bailey, "Contributions to Plant Anatomy", Waltham, Chronica Botanica Co., 1954, 259 p.
- ²⁷ N. Terashima, K. Fukushima, L. F. He and K. Takabe, in "Forage Cell Wall Structure and Digestibility", edited by H. G. Jung, ASA-CSSA-SSSA, 1993, pp. 247-270.