Structure-function relationships during metaphloem sieve elements development in *Triticum aestivum*

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Abstract

The differentiation of metaphloem sieve element (MSEs) in the developing caryopsis of wheat (*Triticum aestivum* L.) was a programmed cell semi-death process. We studied the changes of microtubules and polysaccharide contents during MSEs development. Some significant features are presented in MSEs, such as cell wall non-uniform thickening, chromatin condensation and so on. During the period of MSEs differentiation, numerous microtubules are distributed in the vicinity of the cell wall, but finally they vanished in mature MSEs. Large glycoconjugates in the cell wall and small glycoconjugates in the Golgi apparatus were observed in the developing MSEs. Programmed cell death (PCD) ceased in the mature MSEs after 6 d after flowering and higher aggregation of glycoconjugates appeared in the cytoplasm. All of these processes were in tight contact with the cell wall non-uniform thickening during PCD.

Additional key words: cell wall, microtubules, polysaccharides, programmed cell death, wheat.

Introduction

Phloem in angiosperms is array of sieve elements (SEs), each of which is associated with one or a few companion cells (CCs). SE is a key structure in the phloem in vascular plants and plays the central role in the long distance transport of photosynthates (Van Bel 2003).

SE undergoes increased vacuolation, chromatin condensation and nuclear DNA degradation during development, which are the typical characteristics of programmed cell death (PCD; Gaffal and Friedrichs 2007, Lenochová *et al.* 2009). We observed that the differentiation of metaphloem sieve elements (MSEs) in the caryopsis of wheat also was an especial PCD process. The mature MSEs survived PCD; moreover, they had surviving mitochondria and sufficient amount of photosynthates (Wang *et al.* 2008). If cells obtain sufficient energy, they would survive for a long time when mitochondria or other few organelles exist (Van Doorn 2005, Zhou *et al.* 2009). So it is better to describe SE development as "programmed cell semi-death" (Van Bel 2003).

It is demonstrated that diverse activities such as vesicle transport, prediction of the alignment of the cell plate and the orientation of the cellulose wall microfibrils are, to varying degrees, mediated by microtubules (MTs; Xu *et al.* 2009, Yang *et al.* 2009). Previous studies suggest that the changes of direction and density of MTs are involved in protophloem sieve elements (PSEs) differentiation and the cell wall pattern development (Eleftheriou 1987, 1994). If phloem is treated with inhibitors of MTs, both sieve elements (SEs) development and phloem transport would be influenced (Yang 2007).

The cellulose, the main component of the cell wall, is synthesized by a plasma membrane enzyme cellulose synthase (Kimura *et al.* 1999), location of which display tight spatial and temporal coupling with MTs (Alexander *et al.* 2006). MTs always lie parallel to cell long axis of tracheary elements during secondary wall formation. When MTs are disrupted, the site of Golgi vesicle fusion may be altered (Milioni 2001). In addition, large quantities of cell wall polysaccharides are synthesized in the Golgi apparatus including the pectin and

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Abbreviations: CCs - companion cells; DAF - days after flowering; MSEs - metaphloem sieve elements; MTs - microtubules; PCD - programmed cell death; PSEs - protophloem sieve elements; SEs - sieve elements.

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hemicelluloses, which are also the main components of cell wall (Dupree and Sherrier 1998, Marlene 2002).

The aim of our study was to examine the changes of microtubules and polysaccharides in MSEs in the developing caryopsis of wheat. We focused on the relationship between those changes and the cell wall development during MSEs differentiation.

Materials and methods

Wheat (*Triticum aestivum* L.) cv. Huamai 8 caryopses were planted in the experiment field of Huazhong Agricultural University, Hubei Province, China. At the beginning of flowering (0 day after flowering, DAF), the flowering spikelets were tagged with a pen mark on the arista, and the ears were tagged with labels. Spikelet samples were collected daily from 0 to 20 DAF.

Small pieces of vascular tissue in caryopses were fixed in 2.5 % glutaraldehyde, post-fixed in 1 % OsO_4 and, after gradual dehydration in acetone series and gradual resin infiltration, embedded in *SPI-PON 812* resin (*SPI Supplies*, USA), and polymerized overnight at 60 °C (Wang *et al.* 2008).

Semi-thin sections (1 μm) were observed using a fluorescence microscope (*Nikon Eclipse 80i*, Tokyo, Japan) under UV radiation. Ultrathin sections (80 - 90 nm) were examined with a transmission electron microscope (TEM, *H-7650*, *Hitachi*, Tokyo, Japan) after double staining with uranyl acetate and lead citrate. The number of SEs, the long axis, minor axis and the thickness of SEs cell wall were measured on at least 20 randomly selected photographs. Images were processed by *ACDsee Power Pack* software (*ACD Systems*, USA)

Small pieces of vascular tissue were immediately fixed in 4 % paraformaldehyde, washed in 10 % DMSO and embedded in *Tissue-Tek OCT 4583* compound (*Sakura Finetek*, Torrance**,** USA). Sections (8 - 10 μm) were obtained by a freezing microtome (*Leica CM1850*, Bensheim, Germany), blocked with 2 % bovine serum albumin (BSA, *Sigma*, St. Louis, USA), treated with primary mouse monoclonal anti-α-tubulin antibody diluted 1:200 with bovine serum albumin (BSA; *Sigma*), incubated with the FITC-conjugated goat antimouse secondary antibody diluted 1:100 with BSA, and examined with a fluorescence microscope (*Nikon Eclipse 80i*). A control was provided by omission of first antibody step.

Small pieces of vascular tissue were immediately fixed in a mixture of 4 % glutaraldehyde and 3 % paraformaldehyde (Karnovsky 1965), post-fixed in 1 % OsO_4 and, after gradual dehydration in acetone series and gradual resin infiltration, embedded in *SPI-PON 812* resin, and polymerized overnight at 60 °C (Wang *et al.* 2008). Ultrathin sections (80 - 90 nm) were examined with a TEM (*H-7650*) after double staining with uranyl acetate and lead citrate.

Small pieces of vascular tissue were only fixed in 4 % paraformaldehyde and, after gradual dehydration in acetone series and gradual resin infiltration, embedded in *SPI-PON 812* resin, and polymerized overnight at 60 °C (Wang *et al.* 2008). Semi-thin sections (1 μm) were treated with 1 % periodic acid, stained in Schiff's reagent, rinsed in rinsing solution, and then stained in 0.05 % toluidine blue O. Images were acquired using a light microscope (*Nikon Eclipse 80i*).

Sub-celluar localization of polysaccharides was undertaken by using the method of Thiéry (1967). Ultra-thin sections (80 - 90 nm) were collected on 100 mesh with nickel grids covered with a lab-made *Formvar* (0.3 % chloroform) supporting film. Then the sections were floated on 1 % periodic acid, floated on 2 % thiocarbohydrazide in 20 % acetic acid, washed in graded acetic acid series, floated on 1 % silver proteinate (PA-TCH-SP reaction), and finally washed in ddH_2O. A control was provided by omission of PA oxidation step. The dried grids were observed in TEM (*H-7650*).

Results

The cell wall showed blue autofluorescence after UV excitation. SEs were easily identifiable from other phloem cells by their characteristic cell wall with intensive autofluorescence. The number of SEs rapidly increased as well as the area of phloem from 0 to 8 DAF (Fig. 1*a-d*, Table 1). Enough sugar transport channels formed during the period of SEs differentiation.

MSEs at 0 DAF, showed thin cell walls, the characteristic of young plant cells (Fig. 2*a*). From 2 to 4 DAF, MSEs undergone typical PCD. Meanwhile, the cell wall thickened quickly, more conspicuous thickening appeared in corner of the cell. The thickness of the wall reached the peak at 4 DAF (Fig. 2*b-d*). Thereafter, MSEs matured gradually. Further, many cavities formed nearby the plasma membranes and some cavities released electron-dense materials. The cell wall became smooth and the thickness slightly decreased (Fig. 2*e-f*, Table 1).

It was indicated that the position of microtubules changed in different stage of MSEs development. Phloem mother cells were dividing unequally at 0 DAF, and one daughter cell developed into SE. The microtubules distributed in the middle side of two cells or around the

cell wall (Fig. 3*a*). Horizontal and network-like arrangement of microtubules appeared from 2 to 3 DAF (Fig. 3*b-c*). At 4 DAF, the bipolar microtubules of MSEs increased and exceeded lateral wall (Fig. 3*d*). MSEs matured gradually, the array of microtubules became vague (Fig. 3*e*). The fluorescence of microtubules was not easy to be detected in the control (Fig. 3*f*).

In the early stage of MSEs differentiation (2 DAF), microtubules lay close to the plasmalemma without touching it (Fig. 4*a-c*). During further differentiation of MSEs (from 3 to 4 DAF), the entire cell wall thickened markedly and a large number of Golgi vesicles were observed in the cytoplasm. Meanwhile, most of the microtubules distributed along the developing lateral wall, they paralleled to each other and had the same direction as cellulose microfibrils (Fig. 4*d-f*). The microtubules and vesicles were reduced with MSEs maturation and vanished finally.

The polysaccharides were stained red by Schiff' reagent in the cytoplasm of few MSEs from 1 to 4 DAF, and the cell wall had slight or no coloration (Fig. 5*a-b*). At a later stage, the number of MSEs increased; more MSEs were stained bright red (Fig. 5*c*). The cell wall thickening stopped after MSEs matured, the cell wall was not stained red, but the cytoplasm maintained red till 15 DAF (Fig. 5*d*).

The products of PA-TCH-SP reaction were silver granules. At 1 DAF, the developing cell wall of MSEs appeared to consist of growing ridges alternating with depressions, and the silver granules also showed undulating-ribbon structure (Fig. 6*a*). The cell wall

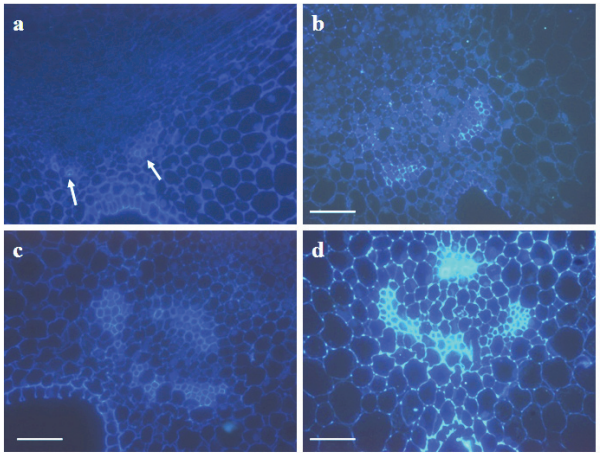

Fig. 1. Light micrographs of the vascular tissue in caryopsis of wheat: *a* - cross-sections of vascular tissue at 0 DAF, a small quantity of SEs appeared (*arrow*); *b* to *d* - the number of SEs increased quickly at 3 - 8 DAF, *bar* = 50 μm.

Table 1. The number of SEs, area of phloem and thickness of MSEs cell wall. Means ± SE, $n = 10$. Area of phloem was calculated as ellipse from measured parameters.

	0 DAF	2 DAF	3 DAF	4 DAF	5 DAF	6 DAF	8 DAF	14 DAF
Number of SEs	5		13			21	27	
Area of phloem [μm^2]	1684.17		5982.86			10425.91	16089.94	
Thickness [μm]	0.13	0.21	0.41	0.61	0.58		0.53	0.52

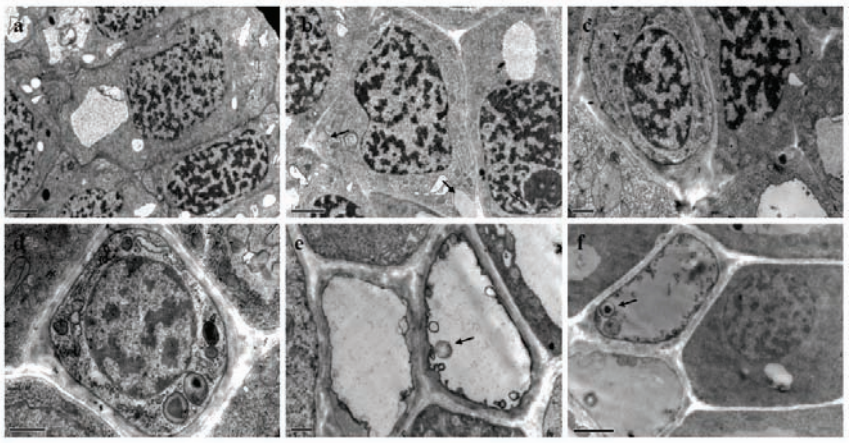

Fig. 2. Ultrastructures of MSEs: *a* - cross-sections of MSEs at 0 DAF, developing MSEs had normal nuclei and thin wall, *bar* = 2 μm; *b,c* - the cell wall thickened, and more conspicuous in corner of cell (*arrows*) at 2~3 DAF, *bar* in *b* = 2 μm, in *c* = 1 μm; *d* - developing MSEs had thickening cell wall, increased chromatin condensation in nucleus at 4 DAF, *bar* 1 μm; *e,f* - some cavities lost integrity and release electron-dense materials in mature MSEs, and the cell wall became smooth (*arrows*) at 8~14 DAF. *bar* in *e* = 1 μm, in *f* = 2 μm.

thickened obviously at 4 DAF and covered with a lot of silver granules; besides, there also were silver granules on the Golgi vesicles and the nucleus (Fig. 6*b-c*). A few aggregations of silver granules appeared in the cytoplasm at 6 DAF (Fig. 6*d-e*) and their number increased till 15 DAF (Fig. 6*g*).

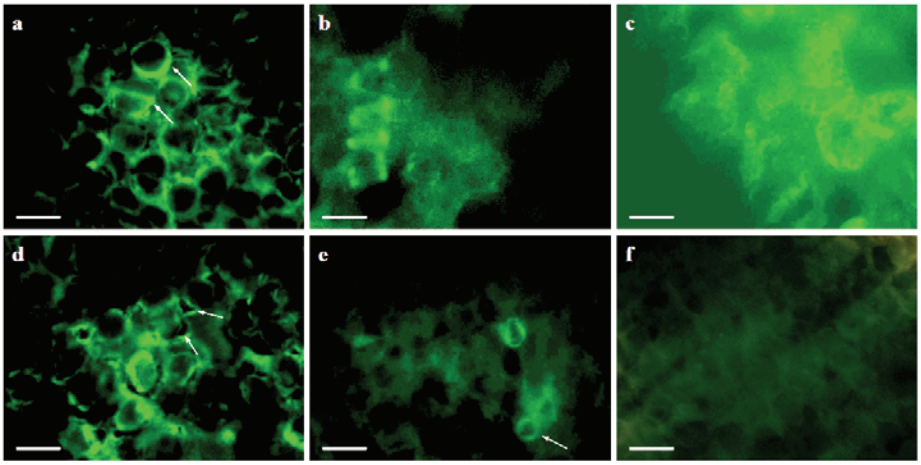

Fig. 3. Fluorescence micrographs of the microtubules. Apart from image *b*, the others are cross-section of phloem. *a* - Microtubules distributed in the middle side of two cells or around the cell wall at 0 DAF; *b* - horizontal arrangement of microtubules appeared at 2 DAF; *c* - network-like arrangement of microtubules appeared at 3 DAF; *d* - the bipolar microtubules of MSEs increased at 4 DAF; *e* - mature MSEs had vague microtubules array at 6 DAF; *f* - control at 4 DAF, microtubule was not observed. *Bars* = 50 μm.

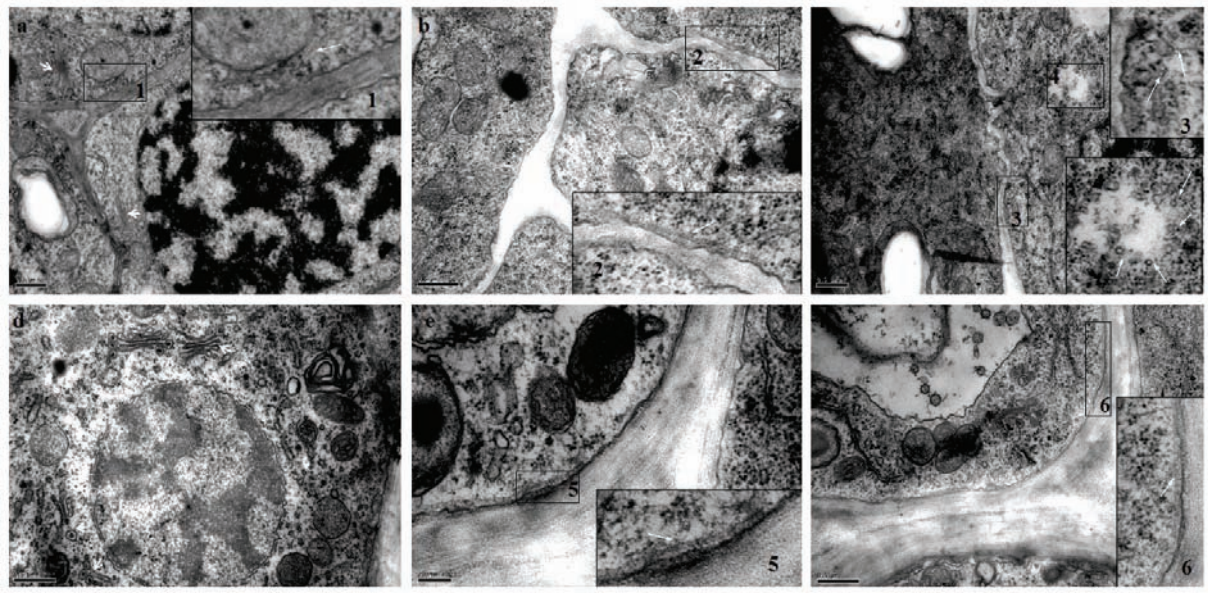

Fig. 4. Transmission electron micrograph of microtubules in MSEs. *Long arrows* denote microtubules, and *short arrows* denote Golgi bodies. Apart from *c*, the others are cross-section of MSEs. *a* - A small number of microtubules (*box area 1*) and Golgi bodies lay close to the plasmalemma at 0 DAF, *bar* = 0.5 μm; *b.c* - most of microtubules distributed in the corner of cell or along the lateral wall (*box areas 2 - 4*) at 2 DAF, *bar* = 0.5 μm; *d* to *f* - most of the microtubules arranged close to the lateral wall (*box area 5,6*) and paralleled to cellulose microfibril, more Golgi vesicles appeared in the cytoplast at 3~4 DAF, *bars* in *d,e* = 200 nm, in *f* = 0.5 μm.

Discussion

After the phloem mother cell divides unequally, one of the daughter cells develops into SE (Ruiz *et al.* 2001). In the early stage of SEs development, we found that the microtubules distributed around the cell division plate where the new wall appeared; thereafter, the horizontal and network-like arrangement of MTs appeared. They might make contribution to the cell wall thickening (Holdaway *et al.* 1995). The MTs form

bands in the definite positions before the wall thickening and so before cellulose deposits in these positions (Milioni 2001). We speculated that the wall material accumulation required the direction of microtubules. Cellulose is synthesized by cellulose synthase, localization of which display tight spatial and temporal coupling to MTs (Alexander *et al.* 2006). MTs are frequently observed to lie parallel to the cellulose fibrils (Zhong *et al.* 2002, Whittington *et al.* 2004). During SEs differentiation, PCD occurred synchronously with distribution of numerous MTs to the corner of the cell. During the cell wall thickening, most of microtubules run parallel to the cell wall. These characteristics explain the phenomenon that more conspicuous thickening appeared in corner of SE wall.

The biosynthesis of plant cell wall requires the trafficking of polysaccharides *via* vesicles. Hemicelluloses and pectin are Golgi-synthesized polysaccharides (Dupree and Sherrier 1998). During the wall thickening, the distribution of microtubules is supposed to direct the Golgi vesicles to reach the wall (Milioni 2001). Many Golgi vesicles transported wall materials to the developing floral nectar glands of *Arabidopsis thaliana* (Zhu 2002). There also were abundant Golgi vesicles and microtubules beside the cell wall when the cell wall of MSEs thickened. It has been proved in previous studies that the cell wall

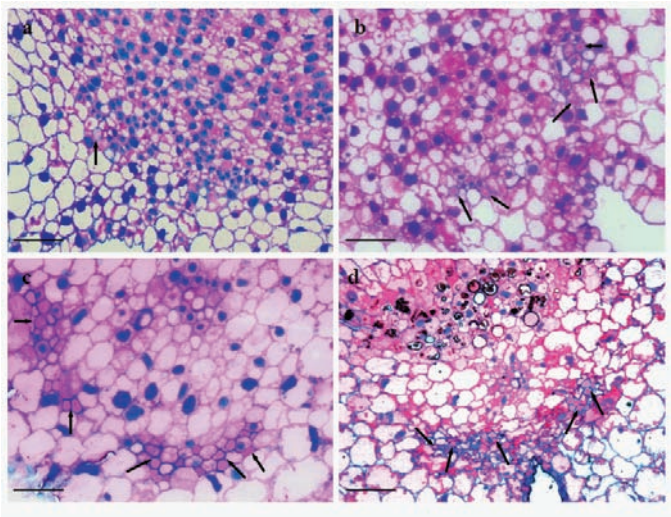

Fig. 5. PAS staining of polysaccharides. Cross-sections of vascular tissue, *arrows* denote coloration of MSEs. *a* - Few MSEs had red cytoplasm at 1 DAF; *b* - more MSEs were painted at 4 DAF; *c* - both MSEs and starch grains of pericarp showed bright red at 6 DAF; *d* - the cytoplasm of mature MSEs still remained red, but the cell wall is without red at 15 DAF. *Bars* = 50 μm.

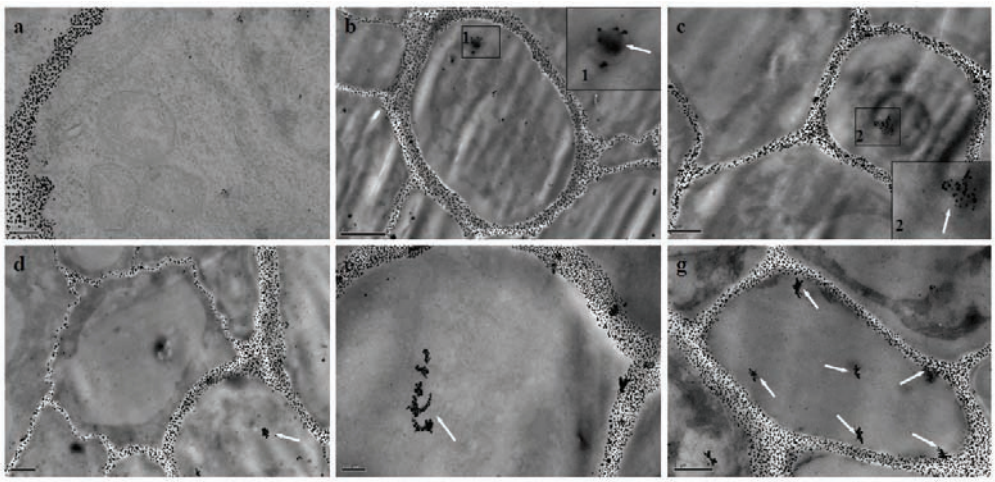

Fig. 6. PA-TCH-SP reaction on polysaccharides. Transmission electron micrographs of cross-sections of MSEs. *a* - The silver granules on the cell wall of MSEs at 1 DAF, *bar* = 200 nm; *b,c* - the number of silver granules increased with the cell wall thickening; small silver granules distributed on the Golgi vesicle (*the boxed area 1*, *arrows*) and the nuclears (*the boxed area 2*, *arrows*) at 4 DAF, *bar* in *b* = 2 μm, in *c* = 1 μm; *d,e* - a few states of aggregation silver granules appeared (*arrow*) at 6 DAF; *bar* in *d* = 1 μm, in *e* = 200 nm; *g* - mature MSEs had abundant states of aggregation silver granules (*arrow*) at 15 DAF, *bar* = 1 μm.

thickening and PCD are two interdependent processes (Groover and Jones 1999, Wang *et al.* 2008). The microtubules vanished and PCD ceased after MSEs maturation. Golgi body and vesicles vanished at the same time. So the wall materials could not be produced and trafficked. As a result, cell wall ceased thickening.

As the organelles of SEs vanishing gradually, the wall materials will meet the demands of SE development (Danilova and Telepova 1981). Since MSEs matured, the cell wall did not thicken any longer and the thickness even slightly decreased. Then we speculated that some wall materials could most likely be used for maintaining MSEs development at the late stages. Thus it can be seen that the microtubules distribution and the vesicles trafficking are related closely to the cell wall thickening.

References

Alexander, R.P., Christopher, R.S., David, W.E.: Visualization of cellulose synthase demonstrates functional association with microtubules. - Science **312**: 1491-1495, 2006.

Danilova, M.F., Telepova, M.N.: Differentiation of sieve elements of the protophloem and metaphloem in the *Hordeum vulgare* (Poaceae) root. - Bot. Zh. (Leningrad) **66**:169-178, 1981.

Dupree, P., Sherrier, D.J.: The plant Golgi apparatus. - Biochim. biophys. Acta. **1404**: 259-270, 1998.

Eleftheriou, E.P.: Microtubules and cell wall development in differentiating protophloem sieve elements of *Triticum aestivum* L. - J. cell. Sci. **87**: 595-607, 1987.

Eleftheriou, E.P.: Abnormal structure of protophloem sieve-element cell wall in colchicine-treated roots of *Triticum aestivum* L. - Planta **193**: 266-274, 1994.

Gaffal, K.P., Friedrichs, G.J.: Ultrastructural evidence for a dual function of the phloem and programmed cell death in the floral nectary of *Digitalis purpurea*. - Ann. Bot. **99**: 593-607, 2007.

Groover, A., Jones, A.M.: Tracheary element differentiation uses a novel mechanism coordinating programmed cell death and secondary cell wall synthesis. - Plant Physiol. **119**: 375-84, 1999.

Karnovsky, M.J.: A formaldehyde-glutaraldehyde fixation of high osmolarity for use in electron microscopy. - J. cell. Biol. **11**: 137-138A, 1965.

Kimura, S., Laosinchai, W., Itoh, T., Brown, R.M.: Immunogold labeling of rosette terminal cellulose synthesizing complexes in the vascular plant *Vigna angularis*. - Plant Cell **11**: 2075-2085, 1999.

Holdaway, N.J., White, R.G., Overall, R.L.: Is the recovery of microtubule orientation in pea roots depend on the cell wall? - J. cell. Biol. **19**: 913-919, 1995.

Hepler, P.K., Hush, J.M.: Behavior of microtubules in living plant cell. - Plant Physiol. **112**: 455-461, 1996.

Lenochová, Z., Soukup, A., Votrubová, O.: Aerenchyma formation in maize roots - Biol. Plant **53**: 263-270, 2009.

Milioni, D.: Differential expression of cell-wall-related genes during the formation of tracheary elements in the *Zinina* mesophyll cell system. - Plant mol. Biol. **47**: 221-238, 2001.

Marlene, B.: A new set of vesicles in *Giardia lamblia.* - Exp. Parasitol. **102**: 30-37, 2002.

Ruiz, M.R., Xoconostle, C.B., Lucas, W.J.: The phloem as a conduit for inter-organ communication. - Curr. Opin. Plant Biol. **4**: 202-209, 2001.

Thiéry, J.P.: Mise en évidence des polysaccharides sur coupes fines en microscopic électronique. - J. Microsc. **6**: 987-1018, 1967.

Van Bel, A.J.E.: The phloem, a miracle of ingenuity. - Plant Cell Environ. **26**: 125-149, 2003.

Van Doorn, W.G.: Plant programmed cell death and the point of no return. - Trends Plant Sci. **10**: 478-483, 2005.

Wang, L.K., Zhou, Z.Q., Song, X.F., Li, J.W., Mei, F.Z.: Evidence of ceased programmed cell death in metaphloem sieve elements in the developing caryopsis of *Triticum aestivum* L. - Protoplasma **234**: 87-96, 2008.

Whittington, A.T., Vugrek, O., Wei, K.J., Sugimoto, K., Rashbrooke, M.C., Wsteneys, G.O.: MOR1 is essential for organizing cortical microtubules in plants. - Nature **411**: 610-613, 2004.

Xu, P., Liu, D., Jiang, W.: Cadmium effects on the organization of microtubular cytoskeleton in interphase and mitotic cells of *Allium sativum.* - Biol. Plant. **53**: 387-390, 2009.

Yang, C.J., Zhai, Z.X.: Effects of acetylcholine, cytochalasin B and amiprophosmethyl on phloem transport in radish (*Raphanus sativas*). - J. Integr. Plant Biol. **49**: 550-555, 2007.

Yang, G., Jan, A., Komatsu, S.: Characterization of β-tubulin 4 regulated by gibberellins in rice leaf sheath. - Biol. Plant. **53**: 422-428, 2009.

Zhong, R., Buek, D.H., Morrison, W.H.: A kinesin-like protein is essential for oriented deposition of cellulose microfibrils and cell wall strength. - Plant Cell **14**: 3101-3117, 2002.

Zhou, Z.Q., Wang, L.K., Yang, C.N.: Study on programmed cell death and dynamic changes of starch accumulation in pericarp cells of *Triticum aestivum* L. - Protoplasma **236**: 49-58, 2009.

Zhu, J., Hu, Z.H.: Ultrastructural study on dictyosome and its vesicle secretion during development of floral nectary of *Arabidopsis thaliana* L. - J. Tongji Univ. **30**: 500-503, 2002.