

**Cell wall, cytoskeleton and signalling molecules involved in plant cell morphogenesis**

HABILITATIONSSCHRIFT  
(SYNOPSIS)

zur Erlangung der *Venia legendi* für das Fach

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## List of selected publications related to Habilitation:

### *Book chapters*

1. **Šamaj J, Boudet AM (2002a)**: Immunolocalisation of enzymes of lignification. In: Chaffey NJ (ed): Wood formation in trees: developmental cell biological techniques. Taylor and Francis, London, New York, pp. 203-216
2. **Šamaj J, Ensikat HJ, Barthlott W, Volkmann D (2002b)**: Immunogold-silver scanning electron microscopy using glycerol liquid substitution. In: Hacker GW, Gu J (eds): Gold and silver staining: techniques in molecular morphology. CRC Press, Boca Raton, London, New York, Washington D.C., pp. 223-234

### *Papers in per-reviewed (Current Content) Journals*

3. **Šamaj J, Bobák M, Blehová A, Krištín J, Auxtová-Šamajová O (1995)** Developmental SEM observations of an extracellular matrix in embryogenic calli of *Drosera rotundifolia* and *Zea mays*. *Protoplasma* **186**, 45-49
4. **Hawkins S, Šamaj J, Lauvergeat V, Boudet AM, Grima-Pettenati J (1997)** Cinnamyl alcohol dehydrogenase (CAD): Identification of important new sites of promoter activity in transgenic poplar. *Plant Physiology* **113**, 321-325
5. **Šamaj J, Hawkins S, Lauvergeat V, Grima-Pettenati J, Boudet AM (1998a)** Immunogold localization of cinnamyl alcohol dehydrogenase 2 (CAD2) indicates a good correlation with cell specific activity of CAD2 promoter in transgenic poplar shoots. *Planta* **204**, 437-443
6. **Šamaj J, Baluška F, Volkmann D (1998b)** Cell specific expression of two arabinogalactan-protein epitopes recognized by monoclonal antibodies JIM8 and JIM13 in maize roots. *Protoplasma* **204**, 1-12
7. **Šamaj J, Baluška F, Bobák M, Volkmann D (1999a)** Extracellular matrix surface network of embryogenic units of friable maize callus contains arabinogalactan-proteins recognized by monoclonal antibody JIM4. *Plant Cell Reports* **18**, 369-374

8. **Šamaj J**, Ensikat HJ, Baluška F, Knox JP, Barthlott W, Volkmann D (1999b) Immunogold- localization of plant surface arabinogalactan-proteins using glycerol liquid substitution and scanning electron microscopy. *Journal of Microscopy* **193**, 150-157
9. **Šamaj J**, Braun M, Baluška F, Ensikat HJ, Tsumuraya Y, Volkmann D (1999c) Specific localization of arabinogalactan-protein epitopes at the surface of maize root hairs. *Plant and Cell Physiology* **40**, 874-883
10. **Šamaj J**, Šamajová O, Peters M, Baluška F, Lichtscheidl I, Knox JP, Volkmann D, (2000) Immunolocalization of LM2 arabinogalactan-protein epitope associated with endomembranes of plant cells. *Protoplasma* **212**, 186-196
11. **Šamaj J**, Ovecka M, Hlavacka A, Lecourieux F, Meskiene I, Lichtscheidl I, Lenart P, Salaj J, Volkmann D, Bögre L, Baluška F, Hirt H (2002c) Involvement of the mitogen-activated protein kinase SIMK in regulation of root hair tip-growth. *EMBO Journal* **21**, 3296-3306
12. **Šamaj J**, Baluška F, Pretova A, Volkmann D (2003a) Auxin deprivation induces a developmental switch in maize somatic embryogenesis involving redistribution of microtubules and actin filaments from endoplasmic to cortical cytoskeletal arrays. *Plant Cell Reports* **21**, 940-945
13. **Šamaj J**, Ovecka M, Hlavacka A, Lecourieux F, Meskiene I, Lichtscheidl I, Lenart P, Salaj J, Volkmann D, Bögre L, Baluška F, Hirt H (2003b) Involvement of MAP kinase SIMK and actin cytoskeleton in the regulation of root hair tip growth. *Cell Biology International* **27**, 257-259
14. **Šamaj J**, Baluška F, Hirt H (2004a) From signal to cell polarity: mitogen-activated protein kinases as sensors and effectors of cytoskeleton dynamicity. *Journal of Experimental Botany* **55**, 189-198
15. **Šamaj J**, Baluška F, Menzel D (2004b) New signalling molecules regulating root hair tip growth. *Trends in Plant Science* **9**, 217-220

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## **1. Lignin and lignification enzymes**

*Appendices 1, 4, 5 (references to publications provided in the appendix are indicated by underlining)*

### **1.1. Introduction**

Lignin is a complex phenolic polymer which has crucial function in mechanical support of the plant body and in plant defence. It is mainly found in the vascular tissues, where its hydrophobicity waterproofs the conducting cells of the xylem and its rigidity strengthens the supporting fiber cells of both xylem and phloem. Moreover, Lignin mixed with suberin is present in the periderm, an outer protective bark layer, of many tree species. In wood pulp production, lignin is not desirable and has to be removed by harsh chemical treatments that are costly in both financial and environmental terms. Much attention has therefore been paid for methods of modifying and/or reducing the lignin content of plants through a genetic engineering approach (for recent review see Boudet et al. 2003). The final steps of lignin precursor biosynthesis depend on two key enzymes, cinnamoyl CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD), both of which are specific for lignin branch biosynthetic pathway. CCR (EC 1.2.1.44) is responsible for conversion of cinnamoyl CoA esters to their corresponding cinnamaldehydes while CAD (EC 1.1.1.95) catalyzes the reduction of these hydroxycinnamaldehydes to give hydroxycinnamyl alcohols (monolignols), the monomeric precursors of lignin. Genetic modifications using an antisense strategy is concentrated on a number of enzymes involved in the biosynthesis of lignins including CCR and CAD. Some potential problems could be avoided by targeting the antisense transgene constructs to selected tissues using tissue-specific promoters.

### **1.2. Tissue-specific expression of cinnamyl alcohol dehydrogenase (CAD)**

CAD is directly involved in lignification and has been purified from the xylem tissue of a number of tree species (Goffner et al., 1992; O'Malley et al., 1992) and in eucalyptus the corresponding mRNA is strongly expressed in the same tissue (Grima-Pettenati et al., 1993). These observations suggested that the CAD promoter would be a good candidate for targeted transgene expression. Although studies on the expression patterns of other genes involved in monolignol synthesis have permitted the expression to be localized to the differentiating xylem (Shufflebottom et al., 1993; Subramaniam et al., 1993; Smith et al., 1994), they have not made the distinction between cells undergoing lignification on the one hand and associated parenchyma cells on the other. The observation made by Feuillet et al. (1995) that

the CAD 2 promoter was apparently not active in lignifying cells (vessels and fibers) but was instead active in adjacent parenchyma cells led them to suggest that the lignification of cell walls occurred through a process of "cell cooperation." In this scenario the monolignols necessary for lignification are produced in associated parenchyma cells, and then exported to the cell undergoing lignification. However, in a subsequent study we have shown (considering our measurements of the length of axially elongated cells in poplar stems) that the application of the standard tissue sectioning method for determining reporter gene expression patterns in herbaceous species is unsuitable for woody plants (Hawkins et al. 1997). Therefore, we developed a new reliable sample preparation technique for analyzing gene expression patterns which takes into account the anatomy of trees. This technique revealed new important sites of CAD 2 promoter activity in stems (Hawkins et al. 1997), shoot apices and roots (Šamaj et al. 1998a) of stable transformed poplar trees. Within stems, the CAD promoter was found to be active in the vicinity of lignifying cells in the bark (phloem fiber cells, sclerids, and periderm), vascular cambium/ differentiating xylem, in the ray cells of the xylem and in the stem pith zone (Fig 1, Hawkins et al. 1997). Activity was visualized as blue colored reaction product by the GUS ( $\beta$ -glucuronidase) assay. Additionally, the activity of the CAD promoter was found in leaf primordia and axils, shell zones, procambium and developing vascular tissues of young leaves and scale leaves (Šamaj et al. 1998a). Scale leaves, which are heavily lignified, play an important role in the mechanical protection of the apical shoot bud. Detailed observations revealed that GUS staining was present within the vascular tissue of scale leaves. This vascular system is composed of large lignified cells surrounded by chambered cells which contain solitary crystals of calcium oxalate and show the strongest GUS activity in their cytoplasm. The outer epidermis of the most laterally located scale leaves is covered with a thick layer of polymeric phenolic material which stains intensively with Safranin O and forms an intact protective barrier (Šamaj et al. 1998a).

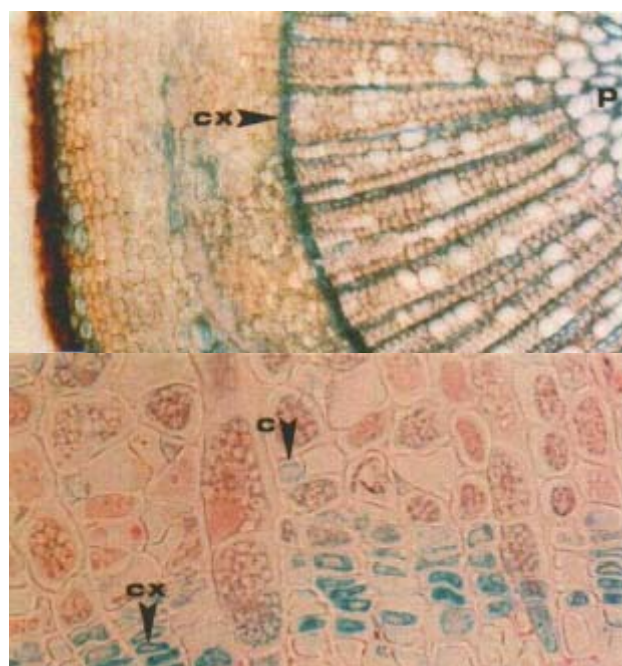
In the shoot apical meristem, no GUS activity was observed within the tunica and flank or rib meristems. In contrast, GUS activity was found in leaf primordia, leaf axils and procambium, as well as in developing vascular tissue of young leaves. Further observations revealed that, in particular, the shell zones of differentiating axillary buds, which are located close to the leaf axils, and procambial strands of cells with developing protoxylem, were most intensively coloured by blue GUS stain (Šamaj et al. 1998a).

Mature roots undergoing secondary growth showed strong GUS activity in the vascular cambium/young xylem area and ray cells, and in phloem fibres, as well as in the phellogen cell layer within the developing root periderm. In young roots, the GUS staining was found in

the young secondary xylem and in the developing cambium. GUS activity was also found in the pericycle, especially during lateral root formation (Fig. 2, Šamaj et al. 1998a). In contrast, no GUS activity was detected in the differentiated protoxylem.

Thus, our results strongly suggest that the CAD 2 promoter is, in fact, also active in cells undergoing lignification and that the lignin monomers necessary for the synthesis of the lignin polymer are produced in both lignifying and parenchyma cells.

Convincing evidence for this hypothesis is provided by the work of Leinhos and Savidge (1993), who localized coniferin (the glycosylated form of the monolignol coniferyl alcohol) in both large and small protoplasts isolated from differentiating xylem of *Pinus banksiana* and *Pinus strobus*. This result suggests that fusiform initials (giving rise to large protoplasts) as well as ray initials (small protoplasts) are capable of storing and, presumably, of synthesizing monolignols.



**Figure 1.** CAD 2 promoter-GUS histochemistry on transverse sections through poplar stems. GUS activity (blue coloration) is present in cortical parenchyma cells, phloem fibers, phloem companion cells (c), and cells interior to periderm, but strongest activity is detected in vascular cambium/differentiating xylem zone (cx), xylem rays and primary xylem/pith zone (p). Lower section is counterstained with Safranin O.

### 1.3. Tissue-specific localization of cinnamoyl-CoA reductase (CCR) and CAD

Immunolocalization techniques revealed that CCR, CAD 1 and CAD 2 (two CAD isoforms) are present in the same cell types in which their corresponding genes are expressed.

Particularly, immunogold/silver labelling regularly showed the localization of CAD 2 in the

shell zone and leaf axils, as well as in the procambial strands and developing protoxylem within the shoot apex (Fig. 2, Šamaj et al. 1998a). Within the xylem zone of mature stems, CCR, CAD 1 and the CAD 2 proteins were immunolocalized to the vascular cambium, young xylem vessels and to the ray parenchyma cells (Šamaj et al. 1998a, Šamaj and Boudet 2002a). Within the phloem, all three enzymes were localized to parenchymatic cells surrounding phloem fibres, but diverse expression patterns were found for other cell types in this tissue. Whereas CCR and CAD 2 were not abundant in companion cells and phloem parenchyma, CAD 1 was moderately expressed in these cell types (Šamaj and Boudet 2002a). On the other hand, CAD 2 was detected only within the lumen of heavily lignified phloem fibres (Šamaj et al. 1998a). Both CCR and CAD 1 were regularly localized to the periderm while the presence of CAD 2 within this tissue was less pronounced (Šamaj and Boudet 2002a). These data suggest that CCR and CAD1 participate on the synthesis of defence lignin which is deposited in the surface cell layers of periderm.

Additionally, immunogold electron microscopy localization, either alone or in combination with silver enhancement, confirmed at the subcellular level the results obtained with light microscopy. It revealed that the CAD 2 protein was present in the cambium/developing xylem area, xylem ray cells and within phloem fibres. Within the young xylem, the silver-enhanced particles were located in the remaining cytoplasm adjacent to the primary cell walls, where deposition of lignin into developing secondary cell walls occurs. The CAD 2 enzyme was unambiguously detected, both with and without silver enhancement of gold particles, in the cytoplasm of cambial and xylem ray cells. Detailed observations showed that part of the immunostaining appeared to be associated with the membranes of the ER and Golgi-derived vesicles, while the remainder was cytosolic (Šamaj et al. 1998a). Such a distribution pattern was regularly observed in cambial cells of transformed as well as non-transformed (control) poplar plants. These results indicate that transformation events as such did not affect the CAD 2 protein distribution.

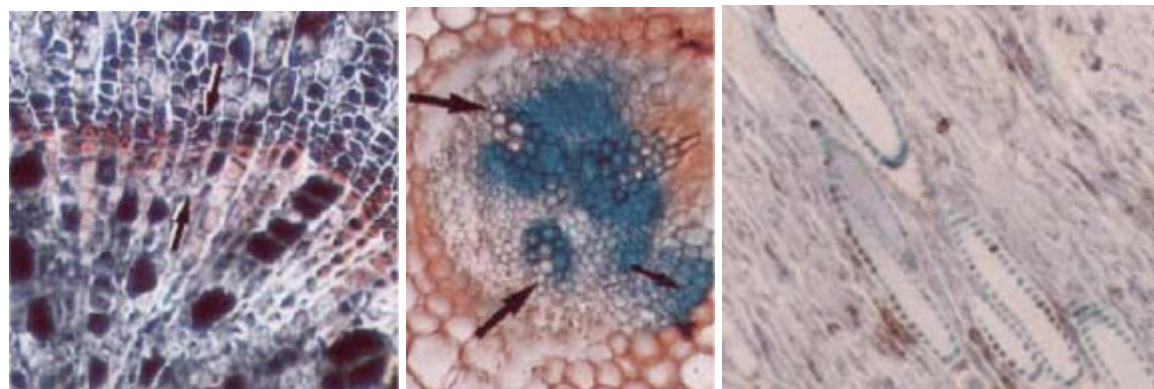


Fig. 2 Histochemistry of GUS and immunogold localization of CAD 2 in roots and shoots of transformed poplar. Root cells with GUS activity have red coloration when observed by dark-field microscopy or blue (indigo) coloration on hand-cut and paraffin-embedded sections when observed by bright-field microscopy. For immunostaining, the sections embedded in LR White resin were treated with polyclonal anti-CAD 2 IgGs and secondary antibody conjugated to colloidal gold. In the bright-field, immunogold-labelled CAD 2 antigen appears as brown-black spots or areas after silver enhancement of gold particles.

In our expression and localization studies, we have shown that the cellular and subcellular fate of the CAD 2 gene translation product correlates well with gene activity during histogenesis in the shoot apex and in mature stems of poplar. Our results indicate that CAD 2 is not expressed in the apical meristem of shoots while it is in the leaf axillary zones, procambium and secondary meristems such as the vascular cambium and phellogen. From a developmental point of view such an observation is interesting, since it indicates that the meristematic cells of the secondary meristems are, in contrast to meristematic cells in the apical meristem, already partially differentiated. While the CAD activity in the root pericycle could be related to the synthesis of 'Casparian strip lignin' (Schreiber 1996), these observations also indicate that the CAD 2 gene is expressed during the early stages of branching events and lateral root formation. Such activity can be necessary to ensure a ready supply of monolignols for subsequent lignification of the developing vascular system. In this respect it is interesting to note that CAD promoter activity was found in trichomes located around axillary buds of transgenic tobacco (Walter et al. 1994) and caffeic acid O-methyl transferase gene promoter activity was found in emerging lateral roots of transgenic maize (Capellades et al. 1996).

Several independent techniques, such as promoter-GUS histochemistry, immunolabelling with specific antibodies, and in situ hybridization, used in an integrated approach, confirmed expression patterns of CAD and CCR related to lignification process in poplar. Our immunolocalization and promoter GUS histochemistry results were fully consistent with the in-situ hybridization data (Hawkins et al. 1997, Lacombe et al. 1997). The subcellular localization of the CAD 2 protein, both to the cytosol and to short elements of ER and Golgi-derived vesicles, raises questions concerning the mechanism of transport of the monolignols to their site of polymerization in the cell wall. In conifers, glucosides of monolignols are accumulated in the vacuole and are presumably transported to the plasma membrane via vesicular trafficking at the appropriate time during xylem differentiation (Whetten and Sederoff 1995). The majority of angiosperms do not accumulate monolignols as glucosides in the vacuole and must therefore use another mechanism for transporting lignin

precursors to the site of polymerization. Work by Smith et al. (1994) on the subcellular distribution of other enzymes of the phenyl-propanoid pathway showed phenylalanine ammonia-lyase (PAL) to be located in cytosol while cinnamate 4-hydroxylase was found to be associated with the ER and Golgi stacks. We have shown the CAD 2 protein to be located in both the cytosol and membranes of ER and Golgi-derived vesicles in monolignol-producing cells. The localization of CAD 2 in the ER and Golgi-derived vesicles correlates well with its role in the synthesis and transport of lignin precursors, while its presence in the cytosol would indicate the existence of another transport mechanism. Importantly, work by Nakashima et al. (1997) showed that there are two different types of both PAL and CAD in tracheary elements derived from *Zinnia* mesophyll cells. One type of each enzyme is distributed in the cytosol, while the other is transported by Golgi-derived vesicles towards the secondary cell walls thickenings of cultured *Zinnia* cells during their lignification. Apparently, further detailed investigation of the extracellular transport of monolignols is necessary, especially in intact plants. Immunogold-cytochemistry employing isoform-specific monoclonal antibodies raised against different types of CAD (CAD 1, CAD 2) seems to be a very promising approach in this respect.

Alltogether, our results support a crucial role for CCR, CAD 1 and CAD 2 in the lignification process and indicate a new role for CAD 2 in branching events within the shoot apex and during lateral root formation.

## **2. Arabinogalactan-proteins**

***Appendices 2,3,6,7,8,9,10 (references to publications provided in the appendix are indicated by underlining)***

### **2.1. Introduction**

Arabinogalactan-proteins (AGPs) represent a subfamily of hydroxyproline rich proteoglycans characterized by high water-holding capacity and inherent stickiness due to their extremely high carbohydrate content and by their overall abundance on cell surfaces. These molecules contain carbohydrate side chains, rich in galactose and arabinose, which are connected to protein backbones rich in Hyp, Ala, Ser, Thr and Gly (Nothnagel, 1997, Showalter 2001). The AGP group of plant proteoglycans is very heterogeneous. Three main criteria for AGP classification have been proposed: first, the presence of arabinogalactan chains, second, a Hyp-rich protein backbone, and third, the ability to bind  $\beta$ -glucosyl Yariv phenylglycoside (a synthetic phenyl-azo dye) (Du *et al.* 1996). However, these criteria as a diagnostic test may be

too restrictive as some AGPs bind little, if any, Yariv phenylglycoside (Nothnagel, 1997). AGPs are associated with plant cell surfaces (Šamaj et al. 1999a,b,c) and they have been found on plasma membranes (Šamaj et al. 2000), within cell walls (Šamaj et al. 1998b), and to be secreted by cells in soluble forms as revealed by biochemical and immunolocalization studies (Knox, 1996). AGPs possess glycosyl-phosphatidyl-inositol (GPI) anchors which are responsible for their localization to the plasma membrane (Youl et al. 1998, Svetek et al. 1999, Shi et al. 2003, Sun et al. 2004). Unfortunately, despite three decades of research on AGPs, the exact functions of these plant specific macromolecules remain enigmatic. Nevertheless, several biological roles for AGPs have been proposed including those in water balance regulation and adhesive events in plants, cell proliferation and cell expansion (Zhu *et al.*, 1993; Serpe and Nothnagel, 1994; Willats and Knox, 1996; Langan and Nothnagel, 1997), and cell signalling in the periplasmic space (Reuzeau and Pont-Lezica, 1995). Exogenous AGPs have been shown to influence somatic embryogenesis (Kreuger and van Holst, 1993, 1995; Egertsdotter and von Arnold, 1995). Many AGP epitopes have shown cell- and tissue-specific expression in roots (Knox *et al.*, 1991; Smallwood *et al.*, 1994, Šamaj et al. 1998b) and flowers (Pennell *et al.*, 1991, 1992). This growing body of evidence suggests that cell surface AGPs play an important role in plant morphogenesis (Knox *et al.*, 1989; Knox, 1997; Nothnagel, 1997, Šamaj et al. 1998b, 1999a,c). Arabinogalactan proteins were also proposed to act as extracellular signalling molecules navigating pollen tubes through the transmitting tract towards the ovules (Cheung et al. 1995) and regulating somatic embryogenic (Mc Cabe et al. 1997). Some AGPs serve as substrates for chitinases, and their cleavage products have promoting effects on somatic embryogenesis (van Hengel et al. 2001). Recently, it was convincingly demonstrated that *Arabidopsis* AGP called Salt Overly Sensistive 5 (SOS5) is essential for proper cell expansion (Shi et al. 2003) while AtAGP30 was implicated to play a role in root regeneration and seed germination (van Hengel and Roberts 2003).

## **2.2. The role of AGPs in somatic embryogenesis**

Embryogenic cultures possess characteristic structural markers known as extracellular matrix surface network. Such networks have been identified by scanning electron microscopy in various plant species (Šamaj et al. 1997) including *Coffea* (Sondahl et al. 1979), *Cichorium* (Dubois et al. 1991, 1992), *Drosera* (Šamaj et al. 1995; Bobák et al. 1995), *Zea* (Šamaj et al. 1995), *Papaver* (Šamaj et al. 1997; Ovecka et al. 1998) and *Fagopyrum* (Rumyantseva et al. 2003). These networks have a fibrillar structure, which was confirmed by transmission electron microscopy in *Zea mays* (Fransz and Schel 1991, 1994, Šamaj et al. 1999a)

embryogenic cultures. It has been suggested that they play an important role in plant morphogenesis (Šamaj et al., 1997). However, the molecular composition of these networks remained elusive. Previously, we described the developmental regulation of extracellular matrix networks in maize embryogenic culture showing that conspicuous layers and networks cover competent embryogenic cells, while no such structures can be seen on the surface of later globular somatic embryos or on non-embryogenic callus cells (Šamaj et al. 1995). We have shown that maize extracellular matrix networks can be efficiently disrupted by protease treatments, indicating that they are, at least partially, of proteinaceous nature. Additionally, these networks can be stabilized by safranin in the fixation mixture, which was shown to preserve proteoglycans in animal cells (Šamaj et al. 1995). These data indicated a proteoglycan nature of such networks. Since the AGPs represent a unique class of plant proteoglycans, we used specific probes against these molecules, employing  $\beta$ -glucosyl Yariv phenylglucoside and monoclonal antibodies raised against AGP epitopes. Both histochemical and immunofluorescence investigations with JIM4 antibody revealed that AGPs are present within the extracellular matrix, which covers embryogenic units of maize friable callus. In contrast to this, surfaces of big non-embryogenic callus cells were depleted of AGP-specific labelling with  $\beta$ -glucosyl Yariv phenylglucoside and JIM4 (Šamaj et al. 1999a). Previously, the JIM4 epitope has been reported to be developmentally expressed during carrot somatic embryogenesis (Stacey *et al.*, 1990). Since we have found the same epitope enriched at the surface of maize embryogenic cultures, a general importance of this AGP epitope during somatic embryogenesis is suggested. Previously, Coimbra and Salema (1997) reported that young zygotic embryos and suspensor cells of *Amaranthus hypochondriacus* were reactive to JIM8, another monoclonal antibody against AGPs. These data together with our observations indicate that the AGPs within the extracellular matrix play an important morphoregulatory role during somatic and zygotic embryogenesis and organogenesis. Thus, in addition to signalling via soluble AGPs (McCabe et al. 1997), AGPs which are integral part of the extracellular matrix are relevant for cell-cell adhesion in embryogenic units as well as for cell-cell signalling and recognition. We proposed that some specific AGP epitopes of the extracellular matrix can participate in the control of cell shape within embryogenic units via preventing their premature elongation and promoting cell proliferation. This concept is supported by the results of Serpe and Nothnagel (1994) showing that precipitation of AGPs with  $\beta$ -glucosyl Yariv phenylglycosides was associated with suppression of cell proliferation in *Rosa* suspension cultures.

### 2.3. The role of AGPs in root development and patterning

The presence of extracellular matrix may have broader relevance for plant morphogenesis. For maize roots, it is known that the extracellular matrix covering young columnar epidermal cells is tripartite and that these three distinct layers differ in their chemical composition and structure (Abeysekera and McCully, 1993). The innermost layer was called the cell wall and the two outer layers are the inner and the outer pellicle. Interestingly, only the outermost layer (outer pellicle) gives strong positive reactions to staining with Coomassie blue and some lectins. This distinct root surface layer stains strongly with  $\beta$ -glucosyl Yariv phenylglucoside (Bacic et al. 1986) indicating its AGP nature. In our work, we have shown that the outer pellicle of maize root contains the AGP epitope MAC207 (Šamaj et al. 1999b). Additionally, we have found the similar extracellular matrix with a fibrillar structure during direct shoot and root organogenesis from flax hypocotyl explants (Šamaj et al. 1997) and we proposed that this extracellular layer might protect meristems both mechanically and via its water-holding capacity. The similar role was proposed for AGP epitopes containing glucuronic acid (LM2) and (1 $\rightarrow$ 6)-galactan (Gal4) during root hair initiation and development in *Zea mays* (Šamaj et al. 1999c).

Several AGPs have been implicated in root patterning in different plant species (Knox *et al.*, 1991; Smallwood *et al.*, 1994, Casero et al. 1998, Šamaj et al. 1998b). We have found that two AGP epitopes JIM8 and JIM13 are structural markers for protophloem sieve elements, while another AGP epitope LM2 was abundant in all other tissues except protophloem sieve elements (Šamaj et al. 1998b).

Additionally, our immunogold electron microscopy studies revealed the association of JIM8 and JIM13 epitopes with plasma membranes and sieve element reticulum, especially on its domains adhered to the plasma membrane (Šamaj et al. 1998b). Other epitopes including LM2 and MAC207 were also localized to the plasma membrane, endoplasmic reticulum, Golgi stacks including Golgi-derived vesicles, and to the tonoplast in plant cells (Šamaj et al. 2000). These data together with biochemical analysis (microsomal association of the same AGP epitopes) suggest that AGPs are tightly associated with endomembranes in plant cells. But what is the function of root AGPs? The recent study on *Arabidopsis* AGP-defective mutant SOS5 shows aberrant cell expansion, especially in root epidermis (Shi et al. 2003) which is reminiscent to the phenotype caused by precipitation of AGPs following treatment with Yariv reagent (Willats et al. 1997). This indicates a role of AGPs in controlled cell expansion during root development. Additionally, another *Arabidopsis* AGP, the AtAGP30, was proposed to play a role in root regeneration (van Hengel and Roberts 2003).

## **2.4. New immunogold/silver SEM method for AGP localization**

Until recently, AGPs have been localized within organs using tissue sections combined with immunofluorescence and immunogold transmission electron microscopy (e.g. Knox *et al.*, 1991; Pennell *et al.*, 1991, 1992; Knox, 1997, Šamaj et al. 1998b, 2000). We developed a new immunogold-SEM method for plant surface antigens and localization of AGP epitopes employing silver-enhanced immunogold labelling in combination with glycerol substitution of the specimen for scanning electron microscopy (SEM) (Šamaj et al., 1999b, c, 2002b). We used our new method in order to study spatial surface distributions of several AGP epitopes on callus and root surfaces. This method has revealed that JIM4 and MAC207 epitopes occur as components of the filamentous structures covering meristematic cells of maize callus and root epidermis. Our immunogold SEM results were supported by immunofluorescence labelling employing the same antibodies on tissue sections from identical plant material. Detailed observations revealed that silver-enhanced particles were linearly arranged, indicating that they decorate filamentous structures both on the callus and on the root surfaces. In conclusion, our new silver-enhanced immunogold SEM method for plant surfaces, based on glycerol substitution, provide sufficient resolution as well as the necessary structural and antigenic preservation of biological samples to be used for other surface antigens of plant and animal cells. Importantly, samples substituted with glycerol do not need metal coating which could mask smaller gold or silver-enhanced gold particles. Thus, gold/silver markers of antigenic sites can be detected with high reliability. This method can be advantageous for the fine immunolocalization studies because fragile thin surface layers and filamentous structures can easily be altered or damaged by other preparation techniques, e.g. by critical point drying conventionally used for SEM (Boyde, 1978) or by immersing specimens in cooling agent for low temperature SEM (Ensikat and Barthlott, 1993).

## **3. The role of cytoskeleton in somatic embryogenesis**

*Appendix 12 (reference to publication provided in the appendix is indicated by underlining)*

### **3.1. Introduction**

Besides cortical microtubules, plant cells also assemble endoplasmic microtubules, which radiate from the nuclear surface towards the periphery of plant cells (for maize root cells see Baluška et al. 1992, 1996). Unlike cortical microtubules, endoplasmic microtubules do not form bundles. The minus ends of these microtubules are attached to the microtubule-organizing nuclear surface (Baluška et al. 1997), whereas the plus ends dynamically explore

the cytoplasmic space. This spatial microtubule arrangement suggests that endoplasmic microtubules might be involved in some sort of signaling between the nucleus and cell periphery (Baluška et al. 1997, 2000). In tip growing plant cells, such as pollen tubes and root hairs, fine networks of microtubules interconnect the subapical nucleus with the apical tip zone, which is enriched with F-actin (Lloyd et al. 1987; Baluška et al. 2000). This feature seems to be essential for the sustained signal-mediated tip growth of these cells (Li et al. 1997; Staiger 2000; Hepler et al. 2001; Šamaj et al. 2004b). Radiating endoplasmic microtubules are prominent in plant cells treated with taxol (Baluška et al. 1997) and inhibitors of both protein synthesis (Mineyuki et al. 1994) and protein kinases/phosphatases (Baskin and Wilson 1997). They are also abundant in *Arabidopsis* mutants with affected cell growth polarity and morphogenesis (Hauser et al. 1995; Traas et al. 1995). Numerous endoplasmic microtubules are present in cells treated with auxin (for maize roots see Baluška et al. 1996) or subjected to diverse stresses including cold, heat (Hause et al. 1993; Gervais et al. 2000), mechanical stress (Caumont et al. 1997), and aluminium (Sivaguru et al. 1999). Similarly, during sporogenesis (Dickinson and Sheldon 1984), endosperm development (Brown et al. 1994), and *Rhizobium* infection (Timmers et al. 1999), centered nuclei organize almost all microtubules in the form of radial arrays. In all these situations, cortical microtubules are usually depleted and/or disassembled. However, little is known about the cytoskeletal organization of embryogenically competent cells during embryogenic induction and subsequent progression (Caumont et al. 1997).

### **3.2. Cytoskeletal re-arrangements in embryogenic cells**

In maize callus, addition of auxin (2,4-D) to the culture medium leads to embryogenic induction of callus cells (Šamaj et al. 1995) accompanied by secretion of a distinct subset of AGPs into the outer cell walls (Šamaj et al. 1999a, b). During this process, nuclei are actively positioned into the centers of embryogenically competent cells. Similarly, the nuclei of embryogenic microspores assume a central position during acquisition of embryogenic competence in these cells (Gervais et al. 2000). Both microtubules and actin filaments radiating from nuclear surfaces are involved in centering of the nuclei and also in maintaining and sensing this central nuclear position via interactions with the cortical cytoskeleton (Grolig 1998; Volkmann and Baluška 1999; Baluška et al. 2000; Collings and Allen 2000). In early embryogenic stages, both induction of cell division and suppression of cell growth are necessary. On the other hand, polarized cell growth is required for advanced embryogenic stages (Šamaj et al. 1995, 1997). Early embryogenic stages (so-called pre-embryogenic units)

are dependent on exogenous auxin, while development of subsequent stages (transition units and somatic embryos) requires auxin deprivation from the culture medium. Exogenous auxin is important for callus cells in order to confer embryogenic potential and to induce mitotic divisions, but auxin deprivation is necessary for further progression of embryogenic morphogenesis. Thus, a developmental switch from non-polar preembryogenic units to polarized transition units in maize embryogenic callus is caused by auxin deprivation from the culture medium. This developmental switch is accompanied by cytoskeletal rearrangements in embryogenic cells (Šamaj et al. 2003a). Our immunofluorescence study revealed prominent endoplasmic microtubules and actin filament meshworks radiating from the nuclear surfaces in pre-embryogenic cells growing on medium supplemented with auxin. On the other hand, parallelly-organized cortical microtubules and cortical actin filament networks are inherently associated with polarized embryogenic cells of transition units growing on medium without auxin (Šamaj et al. 2003a).

If we consider the structural continuity within the cell wall – plasma membrane – cytoskeleton complex, then the above cytoskeletal arrangements of pre-embryogenic cells could be related to their unique cell-to-cell contacts and the composition of cell walls. Embryogenic cells of pre-embryogenic units have loose contacts (Šamaj et al. 1995) and contain AGPs which are believed to have signalling properties (Šamaj et al. 1999a,b). The same cells assemble a prolific endoplasmic cytoskeleton, whereas their cortical cytoskeleton is rather depleted. We proposed that both radiating perinuclear microtubules and actin filaments allow rapid signalling to the nucleus, which enables these cells to respond to changing extracellular stimuli including different levels or absence of exogenous auxin. This suggestion is supported by data from other embryogenic and non-embryogenic systems. For instance, sunflower protoplasts cultured in liquid medium form loosely associated cells and do not embark on the embryogenic pathway. However, when the same cells are embedded in agarose, they start to deposit callose at their regenerating cell walls, assemble numerous perinuclear microtubules, and enter the embryogenic pathway (Caumont et al. 1997). It would be interesting to know, whether these callosic cell walls also accumulate some AGPs. Another example is that rhizobia can trigger the re-arrangement of endoplasmic microtubules in non-dividing outer cortical cells of legume roots (Timmers et al. 1999). Further morphogenesis in maize embryogenic callus is induced after auxin deprivation; the preferred response to this is the assembly of cortical microtubules and, to some degree, actin microfilaments at the peripheries of embryogenic apical cells. Previously, we reported that embryogenic cells of transition units are tightly arranged (Šamaj et al. 1995). Abundant and parallel-oriented

cortical microtubules could, therefore, be important for their tight cell–cell contacts and coordinated cell expansion, which also allows progressive polarization of transition units. In agreement with our data, abundant assembly of cortical microtubules was shown to be characteristic for cells in embryogenic cultures of black spruce (Tautorus et al. 1992). Cortical microtubules were also observed in regions adjacent to the new cross-walls in cellular clumps of alfalfa (Meijer and Simmonds 1988). We conclude that the most critical switch in the embryogenic development from nonpolar to polar structures is accompanied by re-arrangements of cytoskeletal arrays, which might reflect concomitant changes in cell wall organization (Šamaj et al. 1999a, b). These changes are specific for embryogenic cells since they do not occur in non-embryogenic callus cells. Our results indicate that fine-tuning of the dynamic equilibrium between endoplasmic and cortical cytoskeletal arrays is important for polarization and progression of somatic embryogenesis.

#### **4. Mitogen-activated protein kinases and cytoskeleton**

*Appendices 11, 13, 14, 15, 16 (references to publications provided in the appendix are indicated by underlining)*

##### **4.1. Introduction**

Mitogen-activated protein kinases (MAPKs) are one of the best characterized families of signalling molecules in higher plants (Jonak et al., 2002; Šamaj et al. 2004a). Compared with other eukaryotes, plants are equipped with much higher numbers of genes encoding MAPK signalling components. Yeast has six and mammals 13 different MAPKs (Meskiene and Hirt, 2000). In *Arabidopsis*, there are at least 20 MAPK, 10 MAPKK and 60 MAPKKK genes (MAPK group, 2002). In all eukaryotic cells, MAPKs are universal mediators of diverse extracellular signals. MAPKs belong to the serine/threonine class of protein kinases and are involved in a host of crucial cellular responses leading to cell survival, division or differentiation (Garrington and Johnson, 1999). MAPK signalling pathways are built up from dynamic protein complexes involving MAPK modules composed of three kinases organized in a cascade (Fig. 3). In MAPK modules, the MAPKKK, which is also a serine/threonine kinase, phosphorylates MAPKKs which, in turn, perform T and Y dual phosphorylation of MAPKs. In several cases, this basic module is held together through the scaffolding properties of some MAPKKs (e.g. Pbs2 in yeast), MAPKKKs (e.g. MEKK1 in mammals) or specific scaffold proteins (e.g. MP1 and b-arrestins in mammals) (Fig. 3). Apart from scaffolded MAPK modules, other upstream activators, including MAPKKKKs, protein kinase C, small

GTP-ases (Rho, Cdc42, Rac; Rop in plants) and receptor kinases, are important for organizing signalling cascades (Fig. 3). Some of these proteins might also contribute to form signalling complexes of MAPK components with other pathways. Phosphorylation of MAPKs in many cases results in subcellular translocation and subsequent activation of divergent substrate proteins, including transcription factors, other kinases and cytoskeletal proteins. In plants, MAPKs participate substantially in transmitting biotic and abiotic stress, in the control of cell division and developmental processes regulated by hormones and other biologically active compounds, as well as in the plant response to diverse pathogens (Meskiene and Hirt, 2000; Jonak et al., 2002). So far, almost nothing is known about plant scaffolds, upstream regulators of MAPK modules and about molecular targets of MAPKs (Asai et al., 2002; Nishihama et al., 2002; Šamaj et al., 2002c).

The activity and biological output of MAPK signalling pathways is regulated by direct interaction with scaffold proteins and phosphatases. Scaffold proteins are believed to bring specificity into MAPK signalling pathways. Tight control of the subcellular assembly of MAPK components into multiprotein complexes has a significant impact on signalling and is achieved by precise subcellular targeting and recruitment of MAPK modules to various membraneous compartments, for example, the plasma membrane or signalling endosomes. Phosphatases are responsible for the resetting of signalling pathways by dephosphorylation and inactivation of MAPKs (Meskiene et al., 1998). In addition, phosphatases can also tether MAPKs in the cytoplasm or within the nucleus (Mattison et al., 1999), leading to signal termination (Volmat et al., 2001). Importantly, MAPKs, scaffold proteins and phosphatases can shuttle between the nucleus and the cytoplasm.

The cytoskeleton provides structural basis for polarity establishment and maintenance in plant cells (Mathur and Hülskamp 2002; Wasteneys and Galway 2003). In order to accomplish these functions it must be continually re-arranged undergoing cycles of polymerization and de-polymerization of its basic structural subunits (such as G-actin and tubulin dimers). These dynamic changes are believed to be controlled by cytoskeletal proteins such as microtubule-associated proteins (MAPs) and actin-binding proteins (ABPs) (Staiger 2000). During the last decade, it has become obvious that crosstalk between the cytoskeleton and MAPK signalling pathways is important for controlling crucial cellular activities, such as cell division and polarized growth. MAPKs not only regulate the dynamic behaviour of the cytoskeleton via phosphorylation of cytoskeleton-associated proteins, but are also activated themselves by cytoskeletal proteins (e.g. by kinesins or actin) and by changes in the cytoskeletal organization (Šamaj et al. 2004a). However, cytoskeletal targets of activated

MAPKs are unknown in plants and only little is known in other organisms. It seems to be the case that both stimulated and stressed cells use the cytoskeleton as a sensor of changes occurring during cell division or differentiation resulting in turn in the activation of MAPK signalling pathways (Gachet et al., 2001, Šamaj et al. 2002c, 2003b).

Generally, there is considerable similarity in MAPK cascades between mammalian, yeast and plant cells indicating the ubiquitous nature of this type of signalling mechanism (Fig. 3). MAPKs participate in the regulation of a broad range of crucial cellular processes including cell survival, division, polarization, stress responses, and metabolism.

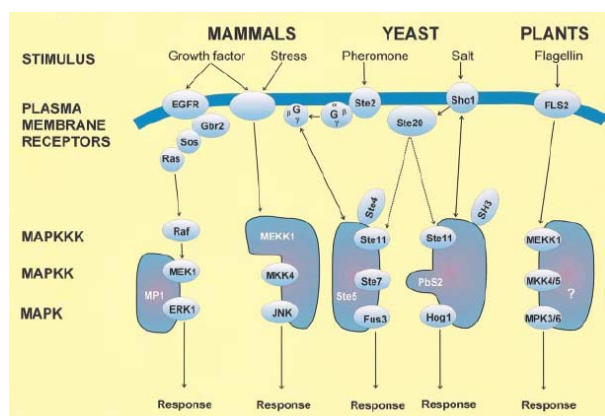


Fig. 3. Scheme of distinct MAPK signalling pathways in mammals, yeast and plants. Note the general similarity in the organization of MAPK pathways in all three eukaryotic systems. MAPKKK, mitogen activated protein kinase kinase kinase; MAPKK, mitogen activated protein kinase kinase; MAPK, mitogen activated protein kinase. Scaffolding proteins (depicted in dark blue) are integrating signalling pathways.

#### 4.2. MAPKs and microtubuli in plants

MAPKs associate with microtubules in plants. For instance, in dividing cells of *Medicago* roots, we observed co-localization of SIMK, a stress-induced MAPK, with mitotic microtubules including pre-prophase bands, phragmoplasts and spindles following stabilization of microtubules by taxol (Fig. 4, Šamaj et al., 2004a) or after cold treatment (Šamaj, Baluška, Ovecka, Hirt, unpublished results). These data indicate that plant mitotic microtubules can interact with SIMK in stressed cells. Recently it was shown that both cold treatment and disruption of the microtubular cytoskeleton by oryzalin activated another stress activated MAP kinase (SAMK) in dividing alfalfa suspension cultured cells (Sangwan et al., 2002). In addition, other plant MAPKs including alfalfa MMK3 and tobacco NtF6 have been localized to the phragmoplast (Calderini et al., 1998; Bögre et al., 1999), a microtubule-based cytoskeletal structure driving cytokinesis of plant cells. Tobacco MAPK kinase kinase NPK1 is essential for cell plate formation and cytokinesis in plant cells (Nishihama et al., 2001).

This kinase binds specifically to the microtubule-associated kinesin NACK1 that is necessary for the activation and transport of NPK1 to the equatorial region of phragmoplasts (Nishihama et al., 2002). In summary, several localization and functional studies in plants indicate that MAPKs can interact with components of the microtubular cytoskeleton, especially in dividing cells.

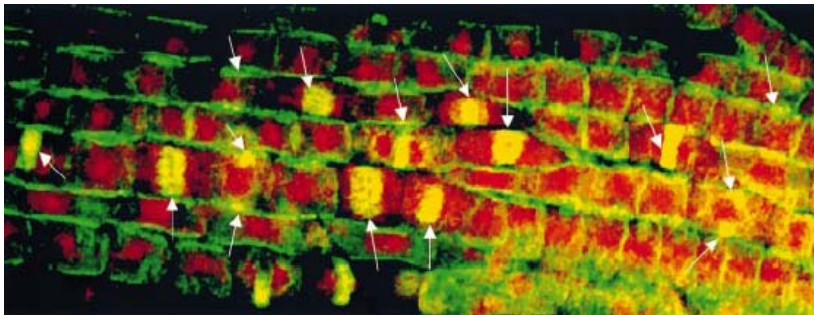


Fig. 4. Immunofluorescence co-localization of microtubules (green, labelled with FITC) and stress-induced MAP kinase SIMK (red, labelled with Texas Red) after taxol treatment of meristematic root cells of *Medicago sativa*. Note the colocalization (yellow, indicated by arrows) of mitotic microtubules (including pre-prophase bands, phragmoplasts and spindles) with SIMK. Cortical microtubules do not colocalize with SIMK.

#### 4.3. MAPKs and actin in plants

In plant cells, disruption of the actin cytoskeleton by latrunculin B causes activation of the alfalfa MAPKs, namely SIMK and SAMK, that are involved in abiotic stress responses including osmotic, heat and cold stress (Šamaj et al., 2002c; Sangwan et al., 2002).

Interestingly, jaspilkinolide, another actin drug, which decreases actin turnover and dynamics, also activates SIMK (Šamaj et al., 2002c) but not SAMK (Sangwan et al., 2002). Conversely, UO126, an inhibitor of mammalian MAPK signalling, causes remodelling of the actin cytoskeleton in plant cells (Šamaj et al., 2002c). These pharmacological data indicate that MAPKs are involved in the dynamic organization of the actin cytoskeleton. In the activated form, MAPKs probably bind to and regulate components of the actin cytoskeleton. On the other hand, disturbances to the actin dynamics and organization are sensed via MAPK pathways. These mutual interactions highlight the importance of both signalling components, i.e., MAPKs and the dynamic actin cytoskeleton in plant cells.

#### 4.4. Root hairs as model for polarity studies

Root hairs are tip-growing outgrowths emerging from hair-forming epidermal cells (trichoblasts) along the length of the root. These polarly-organized exploratory tubes grow exclusively at their apical domes (tip growth); they facilitate water and nutrient uptake from

the soil into the plant and help to anchor the plant body in the soil. Initiation and maintenance of polarity in growing root hairs is under genetic, hormonal (auxin, ethylene) and environmental control (Carol and Dolan 2002). During the last decade, root hairs have emerged as an exciting model system to uncover general principles underlying cell polarity and driving polar growth in plants and other higher eukaryotes.

The tip-focused cytoplasmic calcium ion gradient, the polarly targeted vesicular traffic, and the actin cytoskeleton, represent crucial components of the tip-growth machinery in root hairs (Figs. 5 and 6, Jiang et al. 1997, Wymer et al. 1997, Carol and Dolan 2002, Šamaj et al. 2004b). Overexpression of two actin-binding proteins regulating the dynamic turnover of actin filaments, namely profilin and actin-depolymerizing factor ADF1 resulted in longer or shorter root hairs, respectively (Ramachandran et al. 2000, Dong et al. 2001).

Downregulation of actin-interacting protein AIP1 using RNAi technology resulted in root hair growth inhibition and unusual appearance of thick actin cables ramifying within the root hair tip (Ketelaar et al. 2004), as revealed by *in vivo* observations with the actin marker FABD2 (actin binding domain 2 of *Arabidopsis* fimbrin) fused to the GFP. Moreover, recent genetic studies demonstrated that ACTIN2 is essential for both root hair initiation and growth (Gilliland et al. 2002, Ringli et al. 2002). Calcium ions regulate both cytoskeletal arrangements and vesicular traffic within growing tip (Fig. 6, Wymer et al. 1997, Jones et al. 2002, Foreman et al. 2003). Finally, generation of both tip-focused F-actin and calcium ion gradients, are believed to be regulated by small Rho GTPases of plants called ROPs. Two members of this family, ROP4 and ROP2 have been localized to root hairs (Molendijk et al. 2001, Jones et al. 2002).

#### **4.5. Cross-talks between mitogen-activated protein kinases and the actin cytoskeleton in plant root hairs**

In plants, it was shown that the correct localization and activity of the stress-induced MAP kinase, SIMK, depends on the intact actin cytoskeleton in growing root hairs of *Medicago sativa* (Šamaj et al., 2002c). Before the onset of root hair formation, most of the SIMK in trichoblasts is located in the nucleus as revealed by immunolabelling and the *in vivo* localization of GFP-tagged SIMK. During root hair formation, SIMK becomes redistributed to growing root hair tips possessing dense meshworks of actin filaments (Baluška et al., 2000; Šamaj et al., 2002). Importantly, SIMK is present in its activated form at root hair tips. Actin drugs which interfere with polymerization rates of F-actin, such as latrunculin B and jasplakinolide, cause growth inhibition and removal of both the F-actin meshwork and SIMK

from tips of root hairs (Šamaj et al., 2002c, 2003b). Latrunculin B depolymerizes F-actin by sequestering G actin monomers from the cellular actin pool (Baluška et al., 2000; Hepler et al., 2001; Vidali et al., 2001). For jasplakinolide, both F-actin stabilization (Sawitzky et al., 1999; Šamaj et al., 2002c) and/or disruption of F-actin arrays due to aberrant polymerization (Sawitzky et al., 1999; Ou et al., 2002) were reported in algal and plant cells depending most likely on the cell type and the drug concentration. Upon jasplakinolide treatment of root hairs, a considerable part of SIMK co-localizes with thick actin cables. Both actin drugs also cause the activation of SIMK in dividing suspension cells. Plants overexpressing the GOF (gain-of-function) variant of SIMK, which is constitutively active, show a phenotype of longer root hairs, which emerge earlier than in control plants. Inhibition of MAPK activity by the inhibitor UO126 results in root hair growth inhibition accompanied by the redistribution of both F-actin and SIMK. Tip-focused, activated SIMK and dynamic actin filaments seem to be essential for sustained root hair growth (Šamaj et al., 2002c, 2003b). Moreover, fluorescence recovery after photobleaching (FRAP) experiments revealed that SIMK is undergoing shuttling between the nucleus and the tip region of growing root hairs. These results suggest that SIMK might sense changes in the cytoskeleton and participate in dynamic maintenance of the balance of forces, which are disturbed during bulge initiation by the local weakening of the cell wall resulting in the outgrowth of root hairs.

Importantly, our data suggest that the actin cytoskeleton can act both as a scaffold and effector for MAPK signalling and potentially also for other signalling cascades. In plant root hairs, it was recently demonstrated that NADPH oxidase produces ROS (reactive oxygen species), which are essential for root hair tip-growth. These ROS activate hyperpolarization-activated calcium ion channels localized at root hair tips, thus leading to calcium ion influx (Foreman et al. 2003). One of the possible upstream activators of NADPH oxidases are ROPs (Rho of plants), small GTPases belonging to the Rho family (Jones et al. 2002, Foreman et al. 2003). Additionally, tip-growth is regulated by phosphoinositide signalling involving phospholipase D (PLD) and phosphatidic acid (PA) (Ohashi et al. 2003, Gardiner et al. 2003). Importantly, ROS and phosphatidic acid represent potent triggers of MAPK signalling (Kovtun et al. 2000, Lee et al 2001, Apel and Hirt 2004, Šamaj et al. 2004b) and small GTPases of the Rho family are modulators of MAPK signalling in mammalian and yeast cells (Chen et al. 2003). Moreover, both PA and ROS signals in root hairs can be integrated via OXI1/AGC2-1 kinase which is involved in root hair tip growth and is able to trigger MAPK cascade involving MPK3 and MPK6 (Anthony et al. 2004, Rentel et al. 2004).

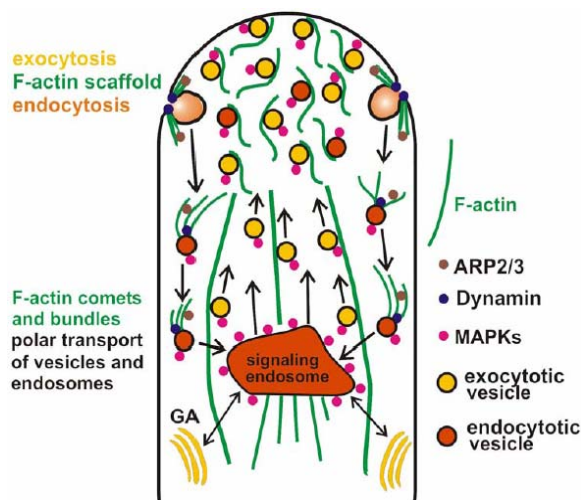


Fig. 5 Working model depicting endosomal/vesicular trafficking and possible roles of the actin filaments in an idealized tip-growing root hair. Local actin polymerization together with accumulation of dynamin could facilitate endocytic recycling of receptors, ion channels and cell wall molecules (e.g. pectins and AGPs), by assisting the pinching off of the endocytic vesicles and by forming actin comets on these vesicles dependent on ARPs. Signaling molecules such as mitogen-activated protein kinases (MAPKs) associate both with endosomal vesicles and the actin cytoskeleton. Additionally, dense meshworks of actin filaments regulated by profilins and actin-related proteins ARPs are suggested to act as a structural scaffold in order to sequester and maintain signaling and regulatory molecules including MAPKs within the apical vesicle pool (“clear zone”). GA = Golgi apparatus. Arrows indicate polar trafficking of exo- and endocytic vesicles/endosomes, as well as putative transport between trans-Golgi network and endosomes.

Recent studies on root hair mutants significantly improved our knowledge on polar growth in plants and revealed that root hair formation is controlled by several signalling pathways including plant hormones auxin and ethylene as well as phosphoinositide-, ROS - and MAPK -dependent signalling pathways. Moreover, available data from plant cells suggest that MAPKs are activated by diverse stimuli including phosphoinositides, ROS and calcium (Jonak et al. 2002, Apel and Hirt 2004) and MAPK signalling pathways have immense potential to integrate all these stimuli into proper cellular response (Šamaj et al. 2002, 2004b).

*OXI1* is a mutant defective in an AGC2-1 kinase gene (this kinase is a downstream target of phosphoinositide-dependent kinase PDK) showing shorter root hairs (Anthony et al. 2004, Rentel et al. 2004). *OXI1* is activated by phosphatidic acid, but also by ROS and cell wall stress (cellulase treatment). MAPKs including *AtMPK3* and *AtMPK6* are downstream of ROS-dependent and cell wall stress signalling cascades (Rentel et al. 2004). Therefore, the *OXI1* can integrate diverse stimuli into a MAPK cascade (Fig.6).

We assume that SIMK, *AtMPK6*, the *Arabidopsis* orthologue of SIMK, and *AtMPK3* will be involved in the transduction of second messenger signals including phosphatidic acid and ROS in root hairs (Fig. 6). Actually, *AtMPK6* was shown to be activated by ROS as well as by diverse abiotic and biotic factors (Kovtun et al. 2000) and SIMK-related MAPK was activated by phosphatidic acid in *Glycine max* (Lee et al. 2001). In view of the close connection between the actin cytoskeleton and signal-transduction (Staiger 2000, Šamaj et al. 2004a), it will be fundamental to learn how all these components of the signalling cascades impinge on the actin cytoskeleton in root hairs. Signalling molecules, including phospholipase D (Ohashi et al. 2003), PA (Goedhard and Gadella, 2004), ROS (Foreman et al. 2003), calcium ions (Wymer et al. 1997) and MAPKs (Šamaj et al. 2002c, 2003b) are all found in root hair tips and can be involved in rearrangements of the actin cytoskeleton via direct interaction with actin and/or with actin-binding proteins such as profilins, ADFs and AIPs. Alternatively, the state of the actin cytoskeleton might regulate the activity of signalling proteins including MAPKs and PLDs, because polymerized filamentous actin was recently shown to enhance the activity of plant PLD *in vitro*, while G-actin has an inhibitory effect (Kusner et al. 2003).

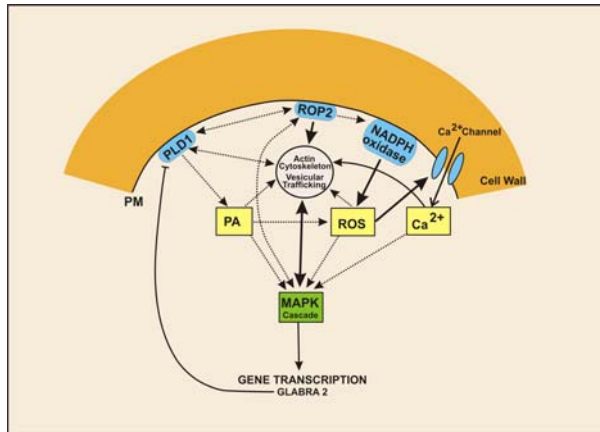


Fig.6 Recent model showing cross-talks between signalling pathways regulating root hair tip growth (adapted from Šamaj et al. 2004b).

ROS (reactive oxygen species) produced by NADPH oxidase activate calcium channels and calcium signalling while *GLABRA2* is a negative transcriptional regulator of PLD-dependent signalling. AGC kinases such as OXI1/AGC2-1 together with mitogen-activated protein kinase (MAPK) cascades are suggested to participate and integrate signal transduction of diverse signals. NADPH oxidase, nicotinamide adenine dinucleotide phosphate oxidase; PA, phosphatidic acid; PLD1, phospholipase D $\zeta$ 1; ROP2, small Rho-like GTPase of plants 2

## 5. Summary

Morphogenesis and control of polarity in plants depends on cell wall molecules, cytoskeleton and signalling. In the work related to this habilitation I describe localizations and potential functions of lignin biosynthetic enzymes, arabinogalactan proteins, actin and microtubular cytoskeleton as well as signalling molecules such as mitogen-activated protein kinases (MAPKs). These molecular components are involved in polar growth and control of cell shape during vessel formation in trees, somatic embryogenesis, root organogenesis and/or in root hair development. The major contributions to better understand plant morphogenesis resulting from this work are the following: i. detection of new sites of gene expression and protein localization for two crucial lignification enzymes CCR and CAD in poplar organs such as stems and roots; ii. identification of arabinogalactan-proteins (AGPs) as the first molecular components appearing in the extracellular matrix which covers surfaces of embryogenic and organogenic plant cells; iii. development of new high resolution technique, based on immunogold localization and scanning electron microscopy for the localization of AGPs on the surface of plant cells including embryogenic cultures, roots and root hairs; iv. localization of AGP epitopes on the subcellular level which revealed their inherent association with plasma membrane, endomembranes and with outer cell walls during root hair development; v. discovery of the dynamic equilibrium between cortical and endoplasmic cytoskeleton (actin microfilaments and microtubuli) as an essential progress in somatic embryogenesis; vi. providing evidence for the involvement of both SIMK, the stress-induced MAPK, and the actin cytoskeleton in polar growth of root hairs.

## 6. Zusammenfassung

### **Die Rolle der Zellwand, des Zytoskeletts und der intrazellulären Signalketten in der pflanzlichen Zellmorphogenese**

Die Kontrolle von Zellpolarität und anderen, komplexeren Zellformveränderungen in Pflanzen ist von einer ganzen Reihe von Proteinfaktoren abhängig, die sich als Elemente der Zellwand, des Zytoskeletts und verschiedener intrazellulärer Signalketten identifizieren lassen. In den Resultaten, die der vorliegenden Synopsi zugrunde liegen, werden besonders die Lokalisation und mögliche Funktion von Enzymen des Ligninstoffwechsel, von Arabinogalaktanproteinen der Zellwand, von Komponenten des Aktin- und Mikrotubulizytoskeletts und besonders auch von Signalmolekülen wie z.B. mitogenaktivierte Proteinkinasen (MAPKs) beschrieben. Diese molekularen Komponenten sind beteiligt an polaren Wachstumsvorgängen, an der Kontrolle der Zellform und Zelldifferenzierung, wie z.B. die Xylemgefäßbildung, an Gewebsbildungsprozessen im Embryo, Organogenese in Wurzel und Spross und an der Ausbildung epidermaler Differenzierungen, wie z.B. Wurzelhaare und Trichome. Durch die hier vorgelegten Arbeiten konnten folgende Beiträge zu diesen Themenbereichen geleistet werden: (i) Die Bestimmung neuer, gewebsspezifischer Orte der Genexpression in Stamm und Wurzel der Pappel von zwei wichtigen Enzymen des Phenylpropanstoffwechsels, die an der sekundären Zellwandmodifikation beteiligt sind. (ii) Die Identifikation eines Arabinogalaktanproteins (AGPs) als eines der ersten makromolekularen Komponenten auf den Oberflächen pflanzlicher Embryonen und den Organoberflächen von Wurzel und Spross junger Keimpflanzen. (iii) Die Entwicklung einer neuen feinstrukturellen Methode zur hochauflösenden, rasterelektronenmikroskopischen Darstellung von AGPs in Kombination mit Immunogold-Markierungstechniken. (iv) Die entwicklungsabhängige, intrazelluläre Lokalisation von AGP-Epitopen an der Plasmamembran, an den Endomembranen und in den äußeren Schichten der Zellwand von Wurzelhaaren. (v) Die Klarstellung der wichtigen Rolle des dynamischen Gleichgewichts zwischen kortikalem und endoplasmatischem Zytoskelett (Aktinfilamente und Mikrotubuli) bei der somatischen Embryogenese. (vi) Die Vorlage von Beweisen dafür, dass SIMKs (stress-induzierte MAPKs) in engem Zusammenspiel mit dem Aktinzytoskelett an der Regulation des Spitzenwachstums in Wurzelhaaren beteiligt sind.

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## 8. *Curriculum vitae*

### Jozef Šamaj

Born on October 3rd, 1964 in Považská Bystrica, Slovak Republic

#### EDUCATION:

- 1993                      **Ph.D. Plant Physiology**  
 Comenius University Bratislava, Slovakia  
 Protoplast culture and transformation of *Helianthus annuus* L.  
 Thesis Advisors: Prof. Dr. Milan Bobák  
    Prof. Dr. Yuri Gleba
- 1987                      **M.Sc. General Biology**  
 Comenius University Bratislava, Slovakia  
 Structural features of opium poppy (*Papaver somniferum* L.) tissue culture  
 Thesis Advisor: Prof. Dr. Milan Bobák

#### ACADEMIC APPOINTMENTS:

- 09/1993 – 11/1996      “Odborný Asistent” (level of Assistant Professor in the anglo-american system)  
 Department of Plant Physiology, Faculty of Natural Sciences,  
 Comenius University, Bratislava, Slovakia
- 12/1996 – 12/2002      **Staff Research Fellow (Senior Researcher)**  
 Institute of Plant Genetics and Biotechnology  
 Slovak Academy of Sciences, Nitra, Slovakia
- 01/2003 - present      **Research Scientist** (Wissenschaftlicher Mitarbeiter BatIIa)  
 Institute of Cellular and Molecular Botany, University of Bonn,  
 Germany

#### HONORS AND AWARDS:

- 1994 – 1995    **Research Fellowship from Eurosilva** (France)  
University of Paul Sabatier Toulouse, laboratory of Prof. Dr. Alain Boudet
- 1996            **Research fellowship from DAAD** (Germany)  
University of Bonn, laboratory of Prof. Dr. Dieter Volkmann
- 1997-1999    **Research fellowship from Alexander von Humboldt Foundation**  
(Germany)  
University of Bonn, laboratory of Prof. Dr. Dieter Volkmann
- 2000-2002    **Marie-Curie Research Fellowship** (EU, Brussel)  
Vienna Biocenter, laboratory of Prof. Dr. Heribert Hirt

#### **REVIEWING ACTIVITIES:**

Protoplasma

*In Vitro* Plant

Journal of Histochemistry and Cytochemistry

#### **BOOK EDITOR**

Šamaj et al. (1997) Structural features of plant morphogenesis in vitro, Veda, Publishing house of the Slovak Academy of Sciences, Bratislava, ISBN 80-224-0505-1

#### **INVITED SPEAKER:**

Deutsche Botanikertagung in Freiburg 2002, symposium on plant cytoskeleton, Sept. 2002

VI International Symposium on Structure and Function of Roots, Stara Lesna, Slovakia, Sept 2003

#### **TEACHING EXPERIENCE:**

1993-1996            “Odborný Asistent” (level of Assistant Professor in the anglo-american system), Department of Plant Physiology, Comenius University Bratislava, 6 semesters of practical courses of Plant Anatomy, Plant Physiology and Plant Cytology, approx. 300 students

1993- 1996            **Thesis advisor**  
two Msc diploma works:  
Henrieta Homolova (defended in May 1994)

Eduard Kolarik (defended in May 1996)

2003- present      *Praktikum - Blockübungen*  
 Konzeption und Durchführung des Blockkurses “Visualisierung von Zellstrukturen mit Hilfe von Reporterproteinen“ (SS 03, WS 03, SS 04)  
 Betreuung von Laborblockstudenten  
*Vorlesung* – aktuelle Forschungsprojekte am Botanischen Institut (einmal pro Semester)  
*Seminare*: Endocytose in pflanzlichen Zellen (Vorschlag)  
*Diplomarbeiten*: Betreuung von 2 zukünftigen Diplomanden im Fach Botanik

***Vortrag im Biologischen Kolloquium zur Vorstellung vor der Fachgruppe Biologie als potentieller Habilitant***

Mitogen-activated protein kinases and actin in root hair tip-growth

University of Bonn, IZMB, 19 April 2004

**Invited oral presentations during the last several years**

Šamaj J (Mai 1998): *Immunolocalization and possible functions of arabinogalactan-proteins during root and callus morphogenesis* (guest of Prof. Paul Knox, Centre for Plant Sciences, University of Leeds, UK)

Šamaj J (February 1999): *Localization of arabinogalactan-proteins in maize roots and callus*, (Hauskolloquium, Botanisches Institut, Universität Bonn, Germany)

Šamaj J (June 1999): *Arabinogalactan-proteins: localization and possible function in signalling during morphogenesis* (guest of Prof. Irene Lichtscheidl, Institut für Pflanzenphysiologie, Universität Wien, Austria)

Šamaj J (Mai 2000): *Immunolocalization of MAPKs in nodules of Medicago sativa* (meeting to EU project co-ordinated by Prof. Ton Bisseling, Wageningen, Netherland)

Šamaj J (September 2002): *MAP kinases and actin cytoskeleton in root hair tip growth* (*Symposium Structure and function of cytoskeleton* (invited speaker, Botanikertagung 2002, symposium on plant cytoskeleton, Freiburg, Germany)

- Šamaj J (October 2002): Involvement of *mitogen-activated protein kinase SIMK in root hair tip growth* (Arabidopsis meeting, seminar for plant biologists from Vienna, Vienna Biocenter, Austria)
- Šamaj J (April 2003): The role of *mitogen-activated protein kinase SIMK in plant root hairs* (Hauskolloquium, Botanisches Institut, Universität Bonn, Germany)
- Šamaj J (September 2003): *Mitogen-activated protein kinase SIMK and actin regulated root hair tip growth* (invited speaker, 6<sup>th</sup> International Symposium on Structure and Function of Roots, Sept 2-6, Stara Lesna, Slovakia)
- Šamaj J (November 2003): *Immunogold localization of arabinogalactan-proteins using SEM and TEM* (invited speaker, EM course for students from TIPNET EU project organized by Prof. Jan Derksen, Nijmegen, Netherland)
- Šamaj J (April 2004): *Mitogen-activated protein kinase SIMK and actin in root hair tip growth* (vorstellung Habilitationsvortrag, Biologisches Kolloquium, Poppelsdorfer Schloss Universität Bonn, Germany)
- Šamaj J (Juni 2004): Regulation of root hair tip growth by stress-induced mitogen-activated protein kinase and the actin cytoskeleton (guest of Prof. Marcelle Holsters, Dept. of Plant Systems Biology, University of Gent, Belgium)

## [9. List of all publications

### Books/Monographs

1. Šamaj J, Bobák M, Ovečka M, Blehová B, Preťová A (1997): Structural features of plant morphogenesis *in vitro*. Veda. Bratislava, pp. 122

### Book chapters

1. Šamaj J, Blehová A, Repčák M, Ovečka M, Bobák M (1999): *Drosera* species (sundew): in vitro culture and the production of plumbagin and other secondary metabolites. In: Bajaj YPS (ed): Biotechnology in agriculture and forestry 43, Medicinal plants XI, Springer Verlag, Berlin, Heidelberg, New York, Tokyo, pp. 105-135

2. Šamaj J, Boudet AM (2002): Immunolocalisation of enzymes of lignification. In: Chaffey NJ (ed): Wood formation in trees: developmental cell biological techniques. Taylor and Francis, London, New York, pp. 203-216

3. Šamaj J, Ensikat HJ, Barthlott W, Volkmann D (2002): Immunogold-silver scanning electron microscopy using glycerol liquid substitution. In: Hacker GW, Gu J (eds): Gold and silver staining: techniques in molecular morphology. CRC Press, Boca Raton, London, New York, Washington D.C., pp. 223-234

4. Ovečka M, Lichtscheidl I, Baluška F, Šamaj J, Volkmann D, Hirt H (2004) Regulation of root hair tip growth: can mitogen-activated protein kinases be taken into account? NATO series (in press)

### Papers in peer-reviewed (Current Contents) Journals

1. Šamaj J, Bobák M, Erdelský K (1988) Plastids in the organogenic callus cells of *Papaver somniferum* L. cultured on various media. *Biologia* **43**, 577-588

2. Bobák M, Šamaj J, Erdelský K (1989) Polymorphism of nuclei and nucleoli in organogenic callus of *Papaver somniferum* L. *The Nucleus* **32**, 1-4

3. Šamaj J, Bobák M, Erdelský K (1990) Histological-anatomical studies of organogenic callus of *Papaver somniferum* L. *Biologia Plantarum* **32**, 14-18
4. Bobák M, Blehová A, Šamaj J, Ovečka M, Krištín J (1993) Studies of organogenesis from the callus culture of sundew (*Drosera spathulata* Labill.). *Journal of Plant Physiology* **142**, 251-253
5. Bobák M, Blehová A, Gajdoš J, Šamaj J (1993) Occurrence, genesis and ultrastructure of anthocyanoplast-like structures in callus cells of *Drosera spathulata* Labill. *Acta Botanica Hungarica* **38**, 221-226
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7. Šamaj J, Auxtová O, Bobák M (1994) Different regeneration potential of various sunflower genotypes (*Helianthus annuus* L.) in meristem culture. *Biologia Plantarum* **36**, 309-311
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9. Šamaj J, Bobák M, Blehová A, Krištín J, Auxtová-Šamajová O (1995) Developmental SEM observations of an extracellular matrix in embryogenic calli of *Drosera rotundifolia* and *Zea mays*. *Protoplasma* **186**, 45-49
10. Bobák M, Blehová A, Krištín J, Ovečka M, Šamaj J (1995) Direct plant regeneration from leaf explants of *Drosera rotundifolia* cultured in vitro. *Plant Cell, Tissue and Organ Culture* **43**, 43-49

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23. Šamaj J, Ensikat HJ, Baluška F, Knox JP, Barthlott W, Volkmann D (1999) Immunogold- localization of plant surface arabinogalactan-proteins using glycerol liquid substitution and scanning electron microscopy. *Journal of Microscopy* **193**, 150-157
  
24. Šamaj J, Bobák M, Kubošníková D, Kolárik E, Krištín J, Ovečka M, Blehová A (1999) Bundle sheath cells are responsible for direct root regeneration from leaf explants of *Helianthus occidentalis* L. *Journal of Plant Physiology* **154**, 89-94
  
25. Baluška F, Šamaj J, Volkmann D (1999) Proteins reacting with cadherin and catenin antibodies are present in maize showing tissue-, domain-, and development-specific associations with endoplasmic-reticulum membranes and actin microfilaments in root cells. *Protoplasma* **206**, 174-187
  
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27. Baluška F, Šamaj J, Napier R, Volkmann D (1999) Maize calreticulin localizes preferentially to plasmodesmata in root apices. *Plant Journal* **19**, 481-488

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29. Bobák M, Hlavačka A, Ovečka M, **Šamaj J** (1999): Effect of trifluralin and colchicine on the extracellular matrix surface networks during early stages of direct somatic embryogenesis of *Drosera rotundifolia* L. *Journal of Plant Physiology* **155**, 387-392
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33. Baluška F, Salaj J, Mathur J, Braun M, Jasper F, **Šamaj J**, Chua N-H, Barlow PW, Volkmann D (2000) Root hair formation: F-actin-dependent tip growth is initiated by local assembly of profilin-supported F-actin meshworks accumulated within expansin-enriched bulges. *Developmental Biology* **227**, 618-632
34. Sivaguru M, Fujiwara T, **Šamaj J**, Baluška F, Yang Z, Osawa H, Maeda T, Mori T, Volkmann D, Matsumoto H (2000) Aluminium-induced 1,3- $\beta$ -D-glucan inhibits cell-to-cell trafficking of molecules through plasmodesmata. A new mechanism of aluminium toxicity in plants. *Plant Physiology* **124**, 991-1006
35. Ovečka M, Bobak M, **Šamaj J** (2000) A comparative structural analysis of direct and indirect shoot regeneration of papaver somniferum L. in vitro. *Journal of Plant Physiology* **157**, 281-289

36. Dedicova B, Hricova A, **Šamaj J**, Obert B, Bobak M, Pretova A (2000) Shoots and embryo-like structures regenerated from cultured flax (*Linum usitatissimum* L.) hypocotyls segments. *Journal of Plant Physiology* **157**, 372-334
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38. **Šamaj J**, Ovecka M, Hlavacka A, Lecourieux F, Meskiene I, Lichtscheidl I, Lenart P, Salaj J, Volkmann D, Bögre L, Baluška F, Hirt H (2002) Involvement of the mitogen-activated protein kinase SIMK in regulation of root hair tip-growth. *EMBO Journal* **21**, 3296-3306
39. Kolisek M, Zsurka G, **Šamaj J**, Weghuber J, Schweyen RJ, Schweigel M (2003) Mrs2p is an essential component of the major electrophoretic  $Mg^{2+}$  influx system in mitochondria. *EMBO Journal* **22**, 1235-1244
40. **Šamaj J**, Baluška F, Pretova A, Volkmann D (2003) Auxin deprivation induces a developmental switch in maize somatic embryogenesis involving redistribution of microtubules and actin filaments from endoplasmic to cortical cytoskeletal arrays. *Plant Cell Reports* **21**, 940-945
41. Baluška F, **Šamaj J**, Menzel D (2003) Polar transport of auxin: carrier-mediated flux across the plasma membrane or neurotransmitter-like secretion? *Trends in Cell Biology* **13**, 282-285
42. Van Gestel, Slegers H, von Witsch, **Šamaj J**, Baluška F, Verbelen J-P (2003) Immunological evidence for the presence of plant homologues of the actin related protein Arp3 in tobacco and maize: subcellular localization to actin-enriched pit fields and emerging root hairs. *Protoplasma* **222**, 45-52
43. **Šamaj J**, Ovecka M, Hlavacka A, Lecourieux F, Meskiene I, Lichtscheidl I, Lenart P, Salaj J, Volkmann D, Bögre L, Baluška F, Hirt H (2003) Involvement of MAP kinase SIMK

and actin cytoskeleton in the regulation of root hair tip growth. *Cell Biology International* 27, 257-259

44. Baluška F, Šamaj J, Wojtaszek P, Volkmann D, Menzel D (2003) Cytoskeleton-plasma membrane-cell wall continuum in plants. Emerging links revisited. *Plant Physiology* 133, 482-491

45. Rumyantseva NI, Šamaj J, Ensikat HJ, Sal'nikov VV, Kostyukova YA, Baluska F, Volkmann D (2003) Changes in the extracellular matrix surface network during cyclic reproduction of proembryonic cell complexes in the *Fagopyrum tataricum* (L.) gaertn callus. *Dokl. Biol. Sci.* 391, 375-378

46. Bobák M, Šamaj J, Hlinková E, Hlavacka A, Ovecka M (2003) Extracellular matrix in early stages of direct somatic embryogenesis in leaves of *Drosera spathulata*. *Biologia Plantarum* 47, 161-166

47. Šamaj J, Baluška F, Hirt H (2004) From signal to cell polarity: mitogen-activated protein kinases as sensors and effectors of cytoskeleton dynamicity. *Journal of Experimental Botany* 55, 189-198

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## **11. Erklärungen**

Ich habe die Habilitationsschrift selbständig verfaßt und keine als die angegebenen Quellen benutzt

Im Rahmen der schriftlichen Habilitationsleistung lege ich 15 Veröffentlichungen vor.

Ich habe anderenorts weder früher noch gleichzeitig Habilitationsverfahren beantragt noch den Titel Dr. habil. verliehen bekommen.

Bonn, im Juni 2004

Dr. Jozef Šamaj

**Cell wall, cytoskeleton and signalling molecules involved in plant cell morphogenesis**

HABILITATIONSSCHRIFT  
(APPENDICES)

zur Erlangung der *Venia legendi* für das Fach

BOTANIK

an der Mathematisch-Naturwissenschaftlichen Fakultät der Rheinischen Friedrich-Wilhelms-  
Universität Bonn

vorgelegt von

**Dr. rer. nat. Jozef Šamaj**

Bonn, im Juni 2004