VERSITA

Cent. Eur. J. Biol. • 8(12) • 2013 • 1172-1182
DOI: 10.2478/s11535-013-0234-5

Central European Journal of **Biology**

The role of chitinases and glucanases in somatic embryogenesis of black pine and hybrid firs

Research Article

Lenka Fráterová[1], Terézia Salaj[2], Ildikó Matušíková[3], Ján Salaj[1,*]

[1]*Department of Cell Biology, Institute of Plant Genetics and Biotechnology, Slovak Academy of Sciences, 951 07 Nitra, Slovak Republic*

[2]*Department of Population Genetics and Breeding, Institute of Plant Genetics and Biotechnology, Slovak Academy of Sciences, 951 07 Nitra, Slovak Republic*

[3]*Department of Molecular Biology and Biotechnology, Institute of Plant Genetics and Biotechnology, Slovak Academy of Sciences, 951 07 Nitra, Slovak Republic*

Received 02 March 2013; Accepted 11 June 2013

Abstract: Glucanase and chitinase enzymes play an important role in different plant processes including defense against pathogens and morphogenesis. Moreover, their role in the processes of somatic embryogenesis has been demonstrated. It has been suggested, that the presence of this type of proteins might be a marker for embryogenic potential of callus cultures. In this work we screened for the presence of glucanases and chitinases in liquid growth media of a set of conifer embryogenic cell lines in order to find correlation with their embryogenic potential. We have found that none of the 12 chitinase isoforms detected in culture media of *Pinus nigra* Arn. or the nine chitinases detected in media with *Abies alba* x *A. cephalonica* and *Abies alba* x *A. numidica* embryogenic tissues could be linked to their embryogenic capacity. Similarly, none of the six glucanase isoforms detected in the extracellular fluid of *Pinus nigra* Arn. cultures can be assigned as a marker of embryogenic potential. Thus, our data indicate the large variability and doubtless importance of glucanases and chitinases for cell growth and development of somatic embryos, however, do not support the premise that they are markers of embryogenesis.

Keywords: *Abies alba x A. cephalonica* • *A. alba x A. numidica* • *Embryogenic potential* • *Liquid culture* • *Pinus nigra* • *PR proteins*

1. Introduction

Extracellular proteins belong to the compounds that participate in the regulation of embryogenesis and originate mostly from cell walls of cultivated plant explants. Chitinases, glucanases [1], peroxidases [2], lipid transferases [3], and arabinogalactan proteins [4] are the most studied extracellular proteins. Chitinases, glucanases and peroxidases are pathogenesis related (PR) proteins, which play an important role in plants against different stress factors and pathogens.

Chitinases are polyglycanhydrolases, which catalyse the hydrolysis of chitin, the main component of the fungal cell wall and the outer skeleton of insects and some sea animals [5]. In normal conditions, chitinases are expressed in low levels in some organs during specific developmental phases, suggesting that some may participate in the processes of development and growth [6-8]. Expression of many chitinases is induced by biotic [9,10] and abiotic stimuli [11]. Therefore, their function in defense mechanisms is well known and documented [12-14].

Many experimental results have proven that extracellular chitinases and glucanases play an important role in the development of gymnosperm [15-19] and angiosperm somatic embryos [20-25]. The regulatory function of chitinases in the process of somatic embryogenesis consists of the ability to

* *E-mail: jan.salaj@savba.sk*

digest arabinogalactan proteins present in cell walls. In consequence, the chemical structure of cell wall polymers is changed as well as the mechanical characteristics – *i.e.* the thickness and size, resulting in a change of direction during cell division [1,26,27]. This hydrolytic ability of chitinases was observed in embryogenic cell lines of *Pinus caribea* [16] and *Daucus carota* [8]. Chitinases can regulate somatic embryogenesis by participating in the production or degradation of lipochitooligosaccharides [28]. While the effect of a chitinase isolated from *Streptomyces griseus* consisted of lipochitooligosaccharide production in embryogenic cultures of *Daucus carota*, a chitinase secreted by somatic cells of *Picea abies* degraded lipochitooligosaccharides in similar cultures [20,29].

Generally, the induction of chitinases is concurrent with the induction of specific *β-1,3*-glucanases [30,31 and others]. *β-1,3*-Glucanases catalyse the hydrolytic cleavage of *β*-1,3-glucan bonds in glucan, which is an important component of plant and fungal cell walls. Glucanases are PR-2 type proteins and are present in all investigated higher plants. Induction of glucanases in plants occurs when plant defence mechanisms against pathogens are activated [10,32-35] and in plant defence reactions induced by wounding, coldness, ozone and UV radiation. Recent studies confirm that these glucanases have a role in many physiological and developmental processes in uninfected plants [5,35], *e.g.* microsporogenesis, cell division, embryogenesis, reproduction, accumulation of storage compounds in the endosperm, fruit ripening and germination of seeds [36]. Glucanases can be localised in vacuoles, or can be secreted by cells into extracellular space [35].

The aim of the present paper was to evaluate the role (if any) of glucanses and chitinases in the process of somatic embryogenesis in an experimental coniferous system.

2. Experimental Procedures

All together, 18 embryogenic cell lines of *Pinus nigra* Arn. and seven embryogenic cell lines of hybrid firs (*Abies alba* x *A. cephalonica* and *A. alba* x *A. numidica*) with different embryogenic potential were used in this study. Embryogenic cell lines were initiated from immature zygotic embryos of *Pinus nigra* Arn. enclosed in megagametophytes on DCR medium [37], supplemented with 2,4-D (2,4-dichlorophenoxy acetic acid, 2 mg dm^{-3}), BAP (6-benzylaminopurine, 0.5 mg dm^{-3}), enzymatic caseinhydrolysate (500 mg dm^{-3}), glutamine (50 mg dm^{-3}), myo-inositol (200 mg dm^{-3}) and sucrose 2%. The medium was solidified with gelrite 0.3%. Medium of the same composition was used for the maintenance of embryogenic tissues. The embryogenic cell lines of hybrid firs were induced and maintained on DCR medium supplemented with BAP (1 mg dm^{-3}), enzymatic caseinhydrolysate (1,000 mg dm^{-3}), glutamine (500 mg dm^{3}) and sucrose 2%, and solidified with gelrite 0.3%. Embryogenic potential of cell lines was characterised on the basis of structural observations and maturation capacity of somatic embryos. Staining with 2% acetocarmine was applied for structural characterization of chosen embryogenic cell lines. Maturation capacity of cell lines was examined on maturation medium DCR supplemented with 6% maltose, 1% gelrite and growth regulators (2,4-D and BAP) were substituted with ABA (abscisic acid, 25 mg dm^{-3}). The embryogenic cell lines were grown on this medium for approximately 2 months. The maturation experiments were repeated twice, with five Petri dishes for each cell line.

After eight cultivation days of chosen embryogenic cell lines on solid DCR medium, 2 g of tissue was resuspended in 25 ml liquid DCR medium (same composition as mentioned above but without gelrite) and cultivated in 100 ml Erlenmeyer flasks on the shaker (100 rpm) in the dark, at 25°C. Sedimented cell volume was measured in each flask after eight days of cultivation in liquid DCR medium. The liquid medium containing the eluate of cultivated cells was taken away, centrifuged for 20 min at 14,000 rpm and 4°C. Supernatant was stored at -80°C. Extracellular proteins present in the supernatants of different embryogenic cell lines were electrophoretically separated on denaturing polyacrylamide gels [38]. Glycolchitin at 1% was added for detection of chitinase activity into the separating gel. Electrophoresis was run at 8°C in a constant electric current of 18 mA (cross section of gel was 109.5 mm^2). When the dye reached the separating gel, the electric current was increased to 24 mA. Concentration of proteins excreted to cultivation media by cultivated tissues was measured in each protein sample using the Bradford method [39]. In preliminary experiments we observed relativaly high enzymatic activity of chitinases and glucanases on agarose disks. In light of this, we monitored extracellular chitinases and glucanases by firstly loading 5 µg of protein into the gels. Following electrophoresis, proteins in the gels were renatured in 50 mmol L^{-1} NaAc (sodium acetate, pH 5.2) and 1% Triton X-100 overnight and then incubated in 0.05 mol L^{-1} NaAc solution (chitinases) or 0.5 mmol L^{-1} NaAc (glucanases) for 1-2 hours at 37°C. Chitinase gels were stained with fluorescent dye (Fluorescent Brightener, 10 mg), in 50 ml 0.05 mol L^{-1} Tris HCl (pH 8.9) and 50 ml H_2O for 15 min at room temperature [40]. Activity of chitinases was detected by UV light. Laminarin

was added into the separation gels for detection of glucanase activity. After renaturing and incubation, gels were fixed in 7% HAc (acetic acid) and 20% MeOH (methanol) for 5 min. Gels were immediately boiled in 1 M NaOH and 0.1% TTC (2,3,5-triphenyltetrazolium chloride) solution for 5-10 min, until the red bands started to appear on the gel. After detection of chitinase and glucanase activities, gels were stained with 0.05% Coomassie Brilliant Blue R-250, 40% MeOH and 10% acetic acid. Molecular weights of separated isoforms of chitinases and glucanases were estimated by comparison with protein ladder (Spectra™ Multicolor Broad Range Protein Ladder 10–260 kDa). The analyses were repeated two to three times for each sample.

3. Results

3.1 Characterisation of embryogenic cell lines

The embryogenic cell lines of *Pinus nigra* (Figure 1a) were characterised by the presence of early somatic embryos that differed in structural organisation and

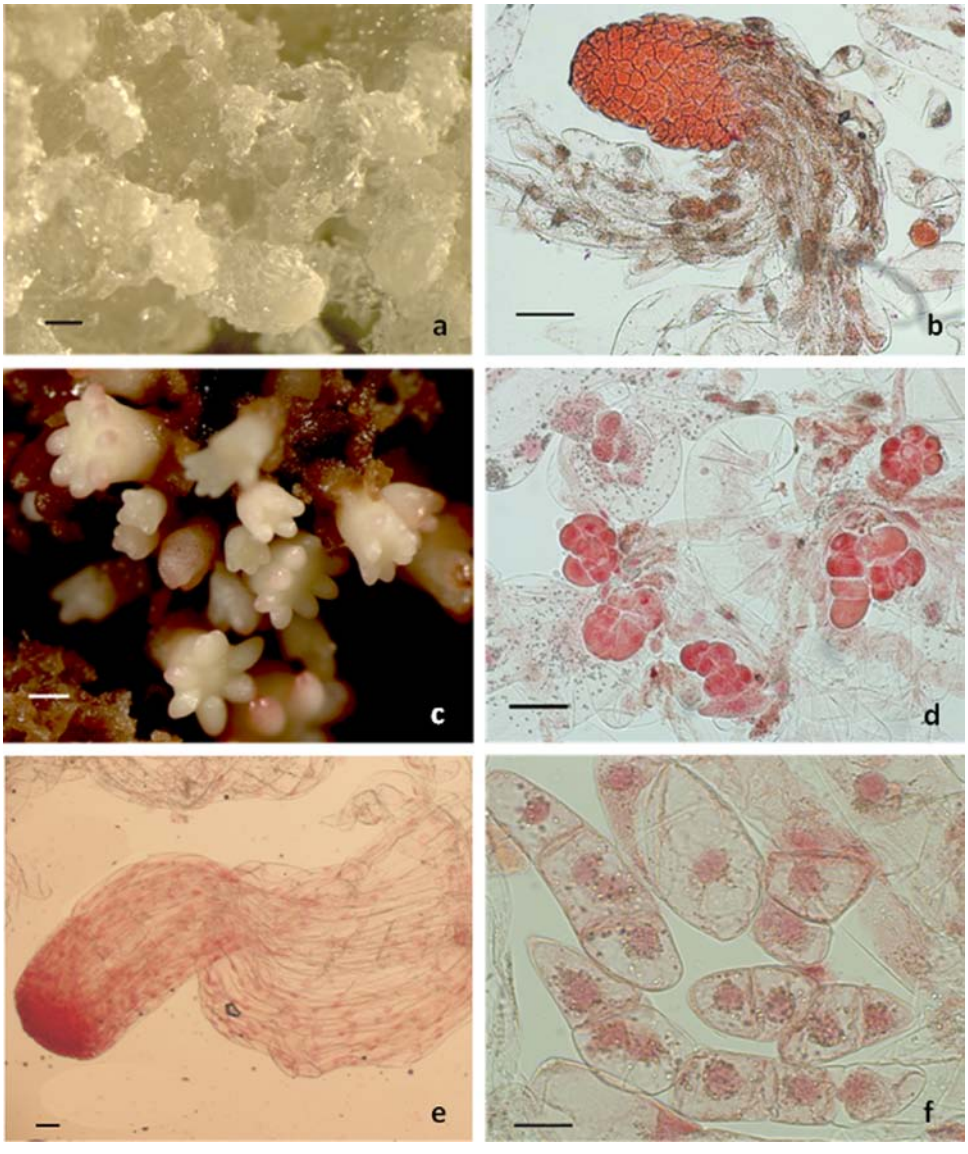

Figure 1. Structural organisation (stained with 2% acetocarmine) of somatic embryos observed in the tissues of the embryogenic cell lines of *Pinus nigra* (a-d) and hybrid fir *Abies alba* x *A. cephalonica* (e, f). a) Embryogenic tissue of *Pinus nigra* on proliferation medium. b) Bipolar somatic embryo with well organised embryogenic part consisted of meristematic cells and suspensor with long vacuolised cells arranged into bundles (*P. nigra* L14). c) Cotyledonary somatic embryos developed on maturation medium. d) Aggregates of meristematic loosely arranged cells without organised suspensor (cell line L72). e) Bipolar structure with well organised embryogenic part consisted of meristematic cells and long vacuolised suspensor cells of embryogenic cell line of hybrid fir *A. alba* x *A. cephalonica* AC1. f) Unorganised cells of cell line AC79. Bars: a, b - 200 μm, c, d, e, f - 100 μm.

maturation capacity depending on cell lines (genotypes). In 12 of analysed cell lines (L14, L68, L69, L71, L73, L76, E263, E266, E323, E324, E325, E326) the embryonal part of somatic embryos had regular outline and consisted of tightly packed meristematic cells subtended by prolonged vacuolised suspensors cells arranged into bundles (Figure 1b). In these cell lines cotyledonary somatic embryos developed (Figure 1c) ranging in number from 35 (E323) to 245 (L68) per 1 g of fresh mass inoculum.The cotyledonary somatic embryos were capable of further development and embling regeneration. These cell lines were designated as cell lines with high embryogenic potential. In cell lines L72, L74, E235, E321, E327 and E329 the early somatic embryos were less organised. The embryonal part was built up as a loosely aggregated mass of meristematic cells and the suspensor cells lacked the organisation into bundles (Figure 1d). These cell lines produced only precotyledonary somatic embryos without emblings regeneration and were designated as cell lines with low embryogenic potential.

The embryogenic cell lines of hybrid *Abies alba x A. cephalonica* were initiated from immature zygotic embryos (AC78 and AC79), mature zygotic embryos (AC1, AC2, AC4) or from cotyledons dissected from seedlings (AC13). The cell line AN72 (hybrid *Abies alba x A. numidica*) was initiated from immature zygotic embryos. The embryogenic tissues of mentioned cell lines (except AC79) contained bipolar somatic embryos (Figure 1e) and produced emblings [41]. In cell line AC79 no bipolar structures were observed (Figure 1f) and no emblings development occurred.

3.2 Detection of chitinolytic activity

Chitinolytic activity was examined in cultivation media of 18 embryogenic cell lines of *Pinus nigra.* There were 12 embryogenic cell lines with high embryogenic capacity (L14, L68, L69, L71, L73, L76, E325, E263, E266, E323, E324, E326) and six with low embryogenic potential (E235, E321, E327, E329, L72 and L74). Separation of proteins originating from cultivation media by SDS-PAGE is shown in Figure 2a. Fractions with chytinolytic activity were quantitatively detected. In most examined embryogenic cell lines (including cell lines with low embryogenic capacity) minimum nine chitinase isoforms with different molecular weights were detected (Figure 2c). In some cell lines, three additional isoforms were present, with approximate molecular weights of 22 kDa, 20 kDa and 16 kDa. Four chitinase isoforms with molecular weights of 90 kDa, 60 kD, 45 kDa and 25 kDa were present in all tested cell lines. The summary of all detected chitinase profiles is presented in Table 1. The comparison of the chitinase profiles of individual embryogenic cell lines shows, that the presence or absence of chitinase isoforms does not correlate clearly with embryogenic potential of tested cell lines.

The chytinolytic activity of the extracellular protein fractions was analysed from the cultivation media of seven hybrid fir embryogenic cell lines: six lines with high embryogenic potential (AC1, AC2, AC4, AC13, AC78, AN72) and one cell line (AC79) with low embryogenic capacity. Separation of proteins originating from cultivation media by SDS-PAGE is shown in Figure 2b. Analysis showed that in most tested embryogenic cell lines at least seven chitinase isoforms with different molecular weights were detected, the exception being the cell line AC79, in which chitinase isoform with molecular weight 40 kDa was missing (Figure 2d). The summary of the detected chitinase profiles of particular cell lines is presented in Table 2.

3.3 Detection of glucanase activity

Glucanase activity was detected from cultivation media of 12 embryogenic cell lines of *Pinus nigra.* From the lines with high embryogenic potential, lines L68, E323, E324, E325, L14, L73, L69, L71, E326 and from among the lines with low embryogenic capacity, lines E235, L72 and L74. Analysis showed that six glucanase isoforms are present, having different abundance in different embryogenic cell lines (Figure 2e). Summary of detected glucanase isoforms is given in Table 3. Profiles of proteins with glucanase activity varied in the embryogenic cell lines of *P. nigra*. In lines E324 and E325, in the 60 kDa molecular weight fraction a stronger glucanase activity was observed in comparison to the other detected isoforms. Similarly, an isoform with a molecular weight of 35 kDa showed strong activity in the embryogenic lines L68 and E326 and a 32 kDa isoform in line L68. On the other hand, in line L73 we did not detect any glucanase isoforms among the proteins present.

Glucanase activity was detected in cultivation media of six hybrid fir embryogenic cell lines (Table 4) with high embryogenic potential AC1, AC2, AC4, AC13, AC78 and AN72. Analysis showed that a glucanase isoform with a molecular weight of 35 kDa was present in all examined embryogenic cell lines with the exception of cell line AC2 (Figure 2f). In the cell line AN72, apart from the mentioned glucanase isoform, we detected another glucanase isoform with a molecular weight of 40 kDa.

4. Discussion

Suspension cultures provide the opportunity to study the role of extracellular molecules secreted into the culture media in the processes of embryo development

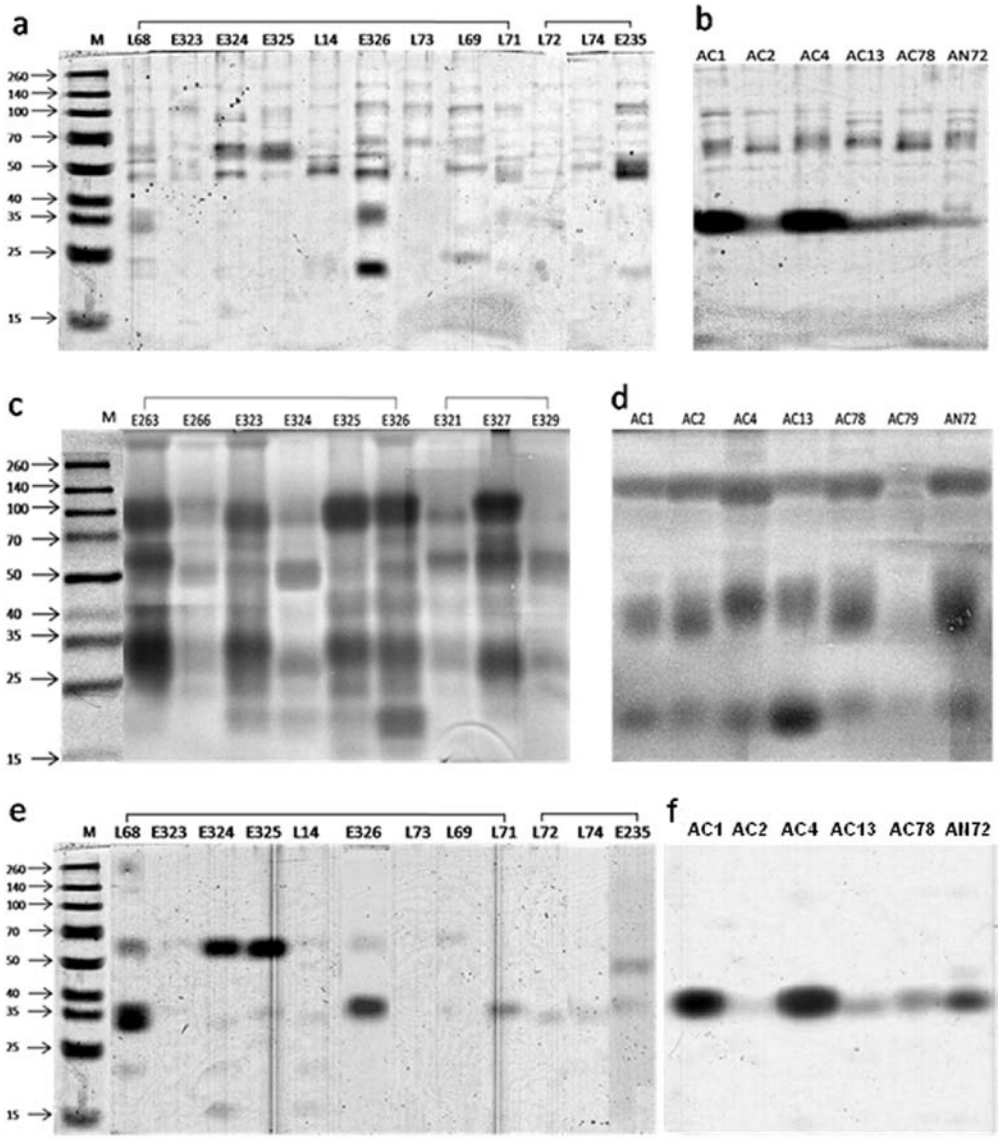

Figure 2. SDS-PAGE separations of complete protein profile of examined a) *Pinus nigra* Arn. embryogenic cell lines (M – marker; cell lines with high embryogenic potential: L68, E323, E324, E325, L14, E326, L73, L69, L71; cell lines with low embryogenic potential: L72, L74, E235) and b) hybrid firs *Abies alba* x *A. cephalonica* and *A. alba* x *A. numidica* embryogenic cell lines (cell lines with high embryogenic potential: AC1, AC2, AC4, AC13, AC78 and AN72). c) Chitinase activity of extracelullar proteins in media of examined *P. nigra* embryogenic cell lines (M – marker; cell lines with high embryogenic potential: E263, E266, E323, E324, E325, E326; cell lines with low embryogenic potential: E321, E327, E329) and d) hybrid firs *A. alba* x *A. cephalonica* and *A. alba* x *A. numidica* embryogenic cell lines (cell lines with high embryogenic potential: AC1, AC2, AC4, AC13, AC78, AN72 and cell line with low embryogenic potential AC79). e) Glucanase activity of extracelullar proteins in media of examined *P. nigra* embryogenic cell lines (M – marker; cell lines with high embryogenic potential: L68, E323, E324, E325, L14, E326, L73, L69, L71; cell lines with low embryogenic potential: L72, L74, E235) and f) hybrid firs *A. alba x A. cephalonica* and *A. alba x A. numidica* embryogenic cell lines (cell lines with high embryogenic potential: AC1, AC2, AC4, AC13, AC78 and AN72).

as well as to identify molecular markers for somatic embryogenesis among these molecules [42].

Brandau *et al.* [43] stated that embryogenic and nonembryogenic suspension cultures of *Euphorbia pulcherrima* can be distinguished on the basis of products secreted into cultivation media. Extracellular arabinogalactan proteins were necessary for carrot somatic embryogenesis [4] and are present as well in embryogenic suspension cultures of *Picea abies* [44]. Some extracellular proteins help to determine the embryogenic potential of plant cells in culture longafter the visible morphological changes have happened

Embryogenic line	Chitinase isoform (kDa)											
	110	90*	70	60*	50	45*	35	30	25*	22	20	16
High EP												
L14	+	+	+	+	+	+	+	+	+	–	+	–
L68	+	+	+	+	+	+	+	+	+	+	–	+
L69	–	+	–	+	+	+	–	+	+	–	+	–
L71	+	+	+	+	+	+	–	+	+	+	–	–
L73	+	+	+	+	+	+	–	+	+	+	–	–
L76	–	+	–	+	+	+	+	+	+	+	–	–
E263	+	+	–	+	+	+	+	+	+	–	+	–
E266	+	+	+	+	+	+	+	+	+	–	–	–
E323	+	+	+	+	+	+	+	+	+	–	–	–
E324	–	+	+	+	+	+	–	+	+	+	–	–
E325	+	+	–	+	+	+	+	–	+	+	–	+
E326	+	+	+	+	+	+	+	–	+	+	+	+
Low EP												
L72	+	+	–	+	+	+	–	+	+	+	–	–
L74	+	+	+	+	+	+	–	+	+	–	–	–
E235	+	+	+	+	+	+	–	+	+	–	–	–
E321	–	+	–	+	–	+	+	+	+	–	–	–
E327	+	+	+	+	+	+	+	+	+	+	–	–
E329	–	+	–	+	+	+	–	+	+	–	–	–

Table 1. Summary of extracellular chitinase profiles in cultivation media of *Pinus nigra* cell lines with different embryogenic potential (EP).

**isoforms present in each sample*

Embryogenic line	Chitinase isoform ~ (kDa)							
	140*	110*	105	50*	45	40	37	22
High EP								
AC1	+	+	–	+	+	+	+	+
AC2	+	+	–	+	–	+	+	+
AC4	+	+	+	+	+	+	–	+
AC13	+	+	–	+	+	+	–	+
AC78	+	+	–	+	+	+	+	+
AN72	+	+	–	+	+	+	+	+
Low EP								
AC79	+	+	–	+	–	–	+	+

Table 2. Summary of extracellular chitinase profiles in cultivation media of hybrid firs (*Abies alba x A. cephalonica* and *Abies alba x A. numidica*) cell lines with different embryogenic potential (EP).

** isoforms present in each sample*

[45]. Until now, some protein markers for embryogenic potential were described [16,24,25,46-51], as well as a structural marker of embryogenic competency and regeneration ability [52]. Two types of protein markers described in the literature are chitinase and glucanase enzymes. Five different isoenzymes of EP3 belonging

to the class IV of plant chitinases, were detected by Kragh *et al.* [53] in carrot embryogenic cultures and one analogous EP3 enzyme was isolated by van Hengel *et al.* [21] from *Arabidopsis* embryogenic suspension cultures. Moreover, Borderies *et al.* [54] detected several kinds of extracellular proteins, including two chitinases (25 and 28 kDa) and one glucanase (30 kDa) in conditioned media of maize microspore cultures. Petrovska *et al.* [55] described chitinases and glucanases as potential markers in flax embryogenic cultures.

In this work, the above premise was studied on a set of conifer embryogenic cell lines of *Pinus nigra*, *Abies alba* x *A. cephalonica* and *Abies alba* x *A. numidica* and characterized by different embryogenic potentials. Preliminary experiments with tissue cultures grown in Petri dishes with a medium containing specific substrates both for glucanases and chitinases confirmed that the tested cultures secreted both types of enzymes with a relatively pronounced activity. The amount of extracellular proteins in the growth media was low (often under the detection limit). We monitored glucanase and chitinase profiles in the cultivation media of 18 cell lines of *P. nigra* and seven cell lines of hybrid firs. These experiments showed that in any given cultivation medium, there were between eight and 11 or between five and eight different chitinase isoforms present in the cases of *P. nigra* and hybrid firs, respectively. Their molecular weight varied in the range typical for plant chitinases, *e.g.* 25-40 kDa [6,56]. Profiles of extracellular chitinases in media were different not only between hybrid firs and *P. nigra*, but also among the cell lines of one species, which reflects the heterogenity of tested cell lines. Domon *et al.* [57] compared profiles of secreted proteins, which were detected on the surface of preglobulary somatic embryos of four embryogenic cell lines of *Pinus caribaea* and those, on the contrary to our results, were very similar. This study was based on literature presenting the hypothesis that chitinases are potential markers for embryogenesis, confirmed by differences in extracellular protein profiles among embryogenic cell lines with different embryogenic capacity [17,58] and also between embryogenic and nonembryogenic cell lines [18,23-25,57,59-61]. However, our results showed that in the 18 embryogenic cell lines of *P. nigra* and the seven cell lines of hybrid firs tested, the presence or absence of any one detected isoform cannot be distinctly associated with the high or low embryogenic potential of the tested cell lines. Even though in the case of hybrid firs this conclusion is still not clearly confirmed due to relatively badly detected profiles and only one nonembryogenic cell line tested, the results from the relatively high number of *P. nigra* lines tested strongly

Embryogenic line	Glucanase isoform (kDa)					
	60	50	35	32	20	15
High EP						
L14	+	–	–	+	+	+
L68	+	–	+	+	+	–
L69	+	–	+	–	–	–
L71	–	–	+	–	–	–
E326	+	–	+	–	–	–
L73	–	–	–	–	–	–
E325	+	–	+	–	–	–
E323	+	–	+	–	–	–
E324	+	–	–	+	+	+
Low EP						
E235	–	+	+	–	–	–
L74	–	–	–	+	–	–
L72	–	–	–	+	–	–

Table 3. Summary of extracellular glucanase profiles in cultivation media of embryogenic cell lines of *Pinus nigra* with different embryogenic potential (EP).

Embryogenic line	Glucanase isoform (kDa)	
	40	35
High EP		
AC1	-	+
AC2	-	-
AC4	-	+
AC13	-	+
AC78	-	+
AN72	+	+

Table 4. Summary of extracellular glucanase profiles in cultivation media of embryogenic cell lines of hybrid firs.

suggests that the qualitative differences of the chitinase profiles are rather the result of the heterogenity of cell lines than a different embryogenic potential. On the other hand, our results can be considered only as semiquantitative, therefore, it cannot be excluded that the activity of some chitinase isoforms present in all protein samples correlates with the embryogenic capacity of different cell lines. Wiweger *et al.* [19] found that the amount of chitinases secreted by embryogenic cell lines of *Picea abies* positively correlated with the embryogenic potential of cell lines. Helleboid *et al.* [23] published similar results: they observed in the medium of the embryogenic cell line of *Cichorium* a two to eight times higher accumulation of extracellular proteins

(*e.g.* chitinases, glucanases and osmotin-like proteins) in comparison to the nonembryogenic cell line. Apparently, the comparison of qualitative and quantitative data concerning chitinase activity, especially their particular isoforms, in sufficiently large pools of tested cell lines allows clear confirmation or disproves the connection with the embryogenic capacity of cell lines. In previous studies it is generally stated that the presence of chitinases in plant tissues is almost always concurrent with the presence of specific glucanases [30,31,62 and others]. In spite of this fact, we detected in the media of embryogenic tissues, unlike to chitinase enzymes, a relatively low number of glucanase isoforms. Their molecular weight varied between extremes typical for plant glucanases (21 to 45 kDa) [63,64]. Besides the analysis of extracellular glucanases in nine *P. nigra* embryogenic cell lines with high embryogenic potential and three embryogenic cell lines with low embryogenic capacity, we detected in particular cell lines a minimum of one and a maximum of four different isoforms from a total number of six. Similarly to the case of chitinases, lower number of glucanases was detected in hybrid firs than in *Pinus nigra*. In the cultivation media of six hybrid firs embryogenic cell lines either none (line AC2), one (in most of cell lines), or two isoforms of extracellular glucanases (lines AC1 and AN72) were detected. The cell line with low embryogenic capacity (AC79) was not analysed for the presence of glucanases. Regardless of the actual glucanase profile of this cell line, qualitative differences in glucanase profiles cannot be connected to embryogenic potential of particular cell lines either. Our results are in accordance with the findings of other authors, *i.e.* an increasing level of extracellular chitinases and glucanases accompanies the process of somatic embryogenesis [23,65] and other processes connected to the development and functioning of reproductive organs [66,67]. Analyses of profiles of extracellular chitinases and glucanases in cultivation media of embryogenic tissues suggest that these profiles are results of the heterogenity of cell cultures and that the probability of a direct correlation between the presence or absence of particular chitinase or glucanase isoforms and embryogenic potential is low. Anyway, the isoenzyme profiles of these enzymes, together with the ratio of accumulation/activity, can be important components of the embryogenic process.

Acknowledgements

We highly appreciate the finantial support from the Slovak Grant Agency VEGA, proj. No. 2-0144-11.

References

[1] de Jong A.J., Cordewener J., Lo Schiavo F., Terzi M., Vandekerckhove J., van Kammen A., et al., A carrot somatic embryo mutant is rescued by chitinase, Plant Cell, 1992, 4, 425-433

[2] Cordewener J., Booij H., van der Zandt H., van Engelen F.A., van Kammen A., de Vries S.C., Tunicamycin-inhibited carrot somatic embryogenesis can be restored by secreted cationic peroxidase isoenzymes, Planta, 1991, 184, 478-486

[3] Sterk P., Booij H., Schellekens G.A., van Kammen A., de Vries S.C., Cell-specific expression of the carrot EP2 lipid transfer protein, Plant Cell, 1991, 3, 907-921

[4] Kreuger M., van Holst G.J., Arabinogalactan proteins are essential in somatic embryogenesis of Daucus carota L., Planta, 1993, 189, 243-248

[5] Kasprzewska A., Plant chitinases – regulation and function, Cell. Mol. Biol. Lett., 2003, 8, 809-824

[6] Collinge D.B., Kragh K.M., Mikkelsen J.D., Nielsen K.K., Rasmussen U., Vad K., Plant chitinases, Plant J., 1993, 3, 31-40

[7] Patil R.S., Ghormade V., Deshpande M.V., Chitinolytic enzymes, Enzyme Microb. Technol., 2000, 26, 473-483

[8] Passarinho P.A., Hengel A.J.V., Fransz P.F., de Vries S.C., Expression pattern of the Arabidopsis thaliana AtEP3/AtchitIV endochitinase gene, Planta, 2001, 212, 556-567

[9] Matušíková I., Salaj J., Moravčíková J., Mlynárová L., Nap J-P., Libantová J., Tentacles of in vitro-grown round-leaf sundew (Drosera rotundifolia L.) show induction of chitinase activity upon mimicking the presence of prey, Planta, 2005, 222, 1020-1027

[10] Moravčíková J., Libantová J., Heldák J., Salaj J., Bauer M., Matušíková I., et al., Stress-induced expression of cucumber chitinase and Nicotiana plumbaginifolia beta-1,3-glucanase genes in transgenic potato plants, Acta Physiol. Plant., 2007, 29, 133-141

[11] Mészáros P., Rybanský Ľ., Hauptvogel P., Kuna R., Libantová J., Moravčíková J., et al., Cultivar-specific kinetics of chitinase induction in soybean roots during exposure to arsenic, Mol. Biol. Rep., 2013, 40, 2127-2138

[12] Popp M.P., Lesney M.S., Davis J.M., Defense responses elicited in pine cell suspension

cultures, Plant Cell Tissue Organ Cult., 1997, 47, 199-206
[13] Brunner F., Stintzi A., Fritig B., Legrand M., Substrate specificities of tobacco chitinases, Plant J., 1998, 14, 225-234
[14] Pirttilä A.M., Laukkanen H., Hohtola A., Chitinase production in pine callus (Pinus sylvestris L.): a defense reaction against endophytes? Planta, 2002, 214, 848-852
[15] Dong J.Z., Dunstan D.I., Molecular biology of somatic embryogenesis in conifers, Molecular Biology of Woody Plants, Vol 1, Kluwer Academic Publishers, Dordrecht, 2000
[16] Domon J. M., Neutelings G., Roger D., David A., David H., A basic chitinase-like protein secreted by embryogenic tissues of Pinus caribaea acts on arabinogalactan proteins extracted from the same cell lines, J. Plant Physiol., 2000, 156, 33-39
[17] Mo L. H., Egertsdottet U., von Arnold S., Secretion of specific extracellular proteins by somatic embryos of Picea abies is dependent on embryo morphology, Ann. Bot., 1996, 77, 143-152.
[18] Domon J., Meyer Y., Faye L., David A., David H., Extracellular (glyco) proteins in embryogenic and non-embryogenic cell lines of Caribbean pine. Comparison between phenotypes of stage one somatic embryos, Plant Physiol. Biochem., 1994, 32, 137-147
[19] Wiweger M.M., Farbos I., Ingouff M., Lagercrantz U., von Arnold S., Expression of Chi4-Pa chitinase genes during somatic and zygotic embryo development in Norway spruce (Picea abies): similarities and differences betwen gymnosperm and angiosperm class IV chitinases, J. Exp. Bot., 2003, 54, 2691-2699
[20] van Engelen F.A., de Vries S.C., Extracellular proteins in plant embryogenesis, Trends Genet., 1992, 8, 66-70
[21] van Hengel A.J., Guzzo F., van Kammen A., de Vries S.C., Expression pattern of the carrot EP3 endochitinase genes in suspension cultures and developing seeds, Plant Physiol., 1998, 117, 43-53
[22] van Hengel A.J., Tadesse A.J., Immerzeel P., Schools H., van Kammen A., de Vries S.C., N-acetylglucosamine and glucosamine-containing arabinogalactan proteins control somatic embryogenesis, Plant Physiol., 2001, 125, 1880-1890
[23] Helleboid S., Hendriks T., Bauw G., Inze D., Vasseur J., Hilbert J.L., Three major somatic embryogenesis related proteins in Cichorium identified as PR proteins, J. Exp. Bot., 2000, 51, 1189-1200
[24] Tchorbadjieva M.I., Kalmukova R., Pantchev I.Y., Kyurkchiev S., Monoclonal antibody against a cell wall marker protein for embryogenic potential of Dactylis glomerata L. suspension cultures, Planta, 2005, 222, 811-819
[25] Tchorbadjieva M.I., Pantchev I.Y., Secretion of chitinase-like protein in embryogenic suspension cultures of Dactylis glomerata L., Biol. Plant., 2006, 50, 142-145
[26] Rea G., Laurenzi M., Tranquilli E., Dóvidio R., Federico R., Angelini R., Developmentally and wound-regulated expression of the gene encoding a cell wall copper amine oxidase in chickpea seedlings, FEBS Lett., 1998, 437, 177-182
[27] Malinowski R., Filipecki M., The role of cell wall in plant embryogenesis. Cell. Mol. Biol. Lett., 2002, 7, 1137-1151
[28] von Arnold S., Bozhkov P., Clapham D., Dyachok J., Filonova L., Hőgberg K. A., et al., Propagation of Norway spruce via somatic embryogenesis. Plant Cell Tissue Organ Cult., 2005, 81, 323-329
[29] Dyachok J.V., Wiweger M., Kenne L., von Arnold S., Endogenous Nod-factor-like signal molecules promote early somatic embryo development in Norway spruce, Plant Physiol., 2002, 128, 523-533
[30] Meins F., Neuhaus J. M., Sperisen C., Ryals J., The primary structure of plant pathogenesis - related glucanohydrolases and their genes, Genes Involved in Plant Defense, Vienna - New York, Springer-Verlag, 1992
[31] Beerhues L., Kombrink E., Primary structure and expression of mRNAs encoding basic chitinase and 1,3-β-glucanase in potato, Plant Mol. Biol., 1994, 24, 353-367
[32] Ashfield T., Hammond-Kosack K.E., Harrison K., Jones J. D. G., Cf gene-dependent induction of a β-1, 3-glucanase promoter in tomato plants infected with Cladosporium fulvum, Mol. Plant-Microbe Interact, 1994, 7, 645-657
[33] Leubner-Metzger G., Fründt C., Vögeli-Lange R., Meins F., Class I ß-1,3-glucanases in the endosperm of tobacco during germination. Plant Physiol., 1995, 109, 751-759
[34] Kini K. R., Vasanthi N. S., Shetty H. S., Induction of β-1,3-glucanase in seedling of pearl millet in response to infection by Sclerospora graminicola, Eur. J. Plant Pathol., 2000, 106, 267-274
[35] Leubner-Metzger G., Functions and regulation of ß-1,3-glucanases during seed germination, dormancy release and after-ripening, Seed Sci. Res., 2003, 13, 17-34
[36] Levy A., Guenoune-Gelbart D., Epel B.L., β-1, 3-glucanases. Plasmodesmal gate keepers for

intercellular communication, Plant Signal Behav., 2007, 2, 404-407
[37] Gupta P.K., Durzan D.J., Shoot multiplication from mature trees of Douglas fir (Pseudotsuga menziessi) and sugar pine (Pinus lambertiana), Plant Cell Rep., 1985, 4, 177-179
[38] Laemmli K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature, 1970, 227, 680-685
[39] Bradford M.M., A rapid and sensitive metod for the quantitation of microgram quantitis of protein utilizing the principle of protein-dye binding, Anal. Biochem., 1976, 72, 248-254
[40] Pan S. Q., Ye X. S., Kuc J., A technique for detection of chitinase, β-1,3-glucanase, and protein patterns after a single separation using polyacrylamide gel electrophoresis or isoelectrofocusing, Phytopathology, 1991, 81, 970-974
[41] Salaj T., Matúšová R., Salaj J., The effect of carbohydrates and polyethylene glycol on somatic embryo maturation in hybrid fir Abies alba x Abies numidica, Acta. Biol. Crac. Ser. Bot., 2004, 46, 159-167
[42] Tchorbadjieva M. I., Protein markers for somatic embryogenesis, Somatic embryogenesis, Plant Cell Monography 2, Berlin - Heidelberg - New York, Springer – Verlag, 2005
[43] Brandau K., Preil W., Lieberei R., Differences in compounds released by embryogenic and non-embryogenic suspension cultures of Euphorbia pulcherrima, Biol. Plant., 1997, 39, 113-124
[44] von Arnold S., Eggersdotter U., Mo L.H., Importance of extracellular proteins for somatic embryogenesis in Picea abies, Current Issues in Plant Molecular and Cellular Biology, Dordrecht – Boston – London, Kluwer Academic Publishers, 1995
[45] Yang X., Zhang X., Regulation of somatic embryogenesis in higher plants. Crit. Rev. Plant Sci., 2010, 29, 36-57
[46] de Jong A.J., Schmidt E.D.L., de Vries S.C., Early events in higher plant embryogenesis, Plant Mol. Biol., 1993, 22, 367-377
[47] Egersdotter U., von Arnold S., Importance of arabinogalactan proteins for the development of somatic embryos of Norway spruce (Picea abies), Physiol. Plant., 1995, 93, 334-345
[48] McCabe P., Valentine T., Forsberg L., Pennell R., Soluble signals from cells identified at the cell wall establish a developmental pathway in carrot, Plant Cell, 1997, 9, 2225-2241
[49] Schmidt E. D. L., Guzzo F., Toonen M. A. J., de Vries S. C., A leucine-rich repeat containing receptor-like kinase marks somatic plant cells competent to form embryos. Development, 1997, 124, 2049-2062
[50] Chugh A, Khurana P., Gene expresion during somatic embryogenesis – recent advances, Curr. Sci., 2002, 83, 715-730
[51] Charbit E., Legavre T., Lardet L., Bourgeois E., Ferriere N., Carron M., Identification of differentially expressed cDNA sequences and histological characterisitcs of Hevea brasiliensis calli in relation to their embryogenic and regenerative capacities, Plant Cell Rep., 2004, 22, 539-548
[52] Šamaj J., Salaj T., Matúšová R., Salaj J., Takáč T., Šamajová O., et al., Arabinogalactan-protein epitope Gal4 is differentially regulated and localized in cell lines of hybrid fir (Abies alba x Abies cephalonica) with different embryogenic and regeneration potential, Plant Cell Rep., 2008, 27, 221-229
[53] Kragh K. M., Hendriks T., de Jong A. J., Lo Schiavo F., Bucherna N., Hojrup P., et al., Characterization of chitinase able to rescue somatic embryos of the temperature-sensitive carrot variant ts11, Plant Mol. Biol., 1996, 31, 631-645
[54] Borderies G., Le Béchec M., Rossigno M., Claude L., Le Deunf E., Beckert M., et al., Characterisation of proteins secreted during maize microspore culture: arabinogalactan proteins (AGPs) stimulate embryo development. Eur. J. Cell Biol., 2004, 83, 205-212
[55] Petrovská B., Salaj T., Moravčková J., Libantová J., Salaj J., Development of embryo-like structures in the liquid cultures of flax coincides with secretion of chitinase-like proteins, Acta Physiol. Plant., 2010, 32, 651-656
[56] Nielsen K. K., Bojsen K., Roepstorff P., Mikkelsen J. D., A hydroxyproline-containing class IV chitinase of sugar beet is glycosylated with xylose, Plant Mol. Biol., 1994, 25, 241-257
[57] Domon J.M., Dumas B., Laine E., Meyer Y., David A., David H., Three glycosylated polypeptides secreted by several embryogenic cell cultures of pine show highly specific serological affinity to antibodies directed against the wheat germin apoprotein monomer, Plant Physiol., 1995, 108, 141-148
[58] Cooutos-Thevenot T., Maes O., Jouenne T., Mauro M.-C., Boulag M., Deloire A., et al., Extracellular protein patterns of grapevine cell suspensions in embryogenic and non-embryogenic situations, Plant Sci., 1992, 86, 137-145
[59] de Vries S.C., Booij H., Janssens R., Vogels R., Saris L., Lo Schiavo F., et al., Carrot somatic embryogenesis depends on the phytohormone

– controlled presence of correctly glycosylated extracellular proteins, Genes Dev, 1988, 2, 462-476

[60] Nielsen K., Hansen I. B., Appearance of extracellular proteins associated with somatic embyogenesis in suspension cultures of barley (Hordeum vulgare I.), J. Plant Physiol., 1992, 139, 489-497

[61] Oropeza M., Marcano A. K., Garcia E. D., Proteins related with embryogenic potential in callus and cell suspensions of sugarcane (Saccharum sp.), In Vitro Cell. Dev. Biol. Plant, 2001, 37, 211-216

[62] Brederode F.T., Linthhorst H.J.M., Bol J.F., Differential induction of acquired resistance and PR gene expression in tobacco by virus infection, ethaphon treatment, UV light and wounding, Plant Mol. Biol., 1991, 17, 1117-1125

[63] Boller T., Antimicrobial functions of the plant hydrolases, chitinase and ß-1,3-glucanase (391-400), Mechanisms of Plant Defense Responses, Kluwer Academic Publishers, Dordrecht, 1993

[64] Meins F., Neuhaus J.M., Sperisen C., Ryals J., The primary structure of plant pathogenesis - related glucanhydrolases and their genes, Mechanisms of Plant Defense Responses, Kluwer Academic Publishers, Dordrecht, 1993

[65] Kragh K.M., Jacobsen S., Mikkelsen J.D., Nielsen K.A., Purification and characterization of three chitinases and one β-1,3-glucanase accumulating in the medium of cell suspension cultures of barley (Hordeum vulgare L.), Plant Sci., 1991, 76, 65-77

[66] Leubner-Metzger G., Meins F., Function and regulation of β-1,3-glucanases PR-2, Pathogenesis-related proteins in plants, CRC Press, Boca Raton, Florida, 1999

[67] Libantová J., Kämäräinnen T., Moravčíková J., Matušíková I., Salaj J., Detection of chitinolytic enzymes with different substrate specificity in tissues of intact sundew (Drosera rotundifolia L.), Mol. Biol. Rep., 2009, 851–856