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Protein Markers for Somatic Embryogenesis

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Abstract The capacity for somatic embryogenesis is a remarkable property of plant cells. Somatic embryogenesis is the process by which somatic cells develop into plants through characteristic morphological changes, thus rendering it a good model system for studying early plant development. Most of the important crops and grasses are recalcitrant for in vitro culturing, which hampers the development of reliable regeneration techniques. Better understanding of the fundamental processes that trigger and control somatic embryogenesis will lead to more rational regeneration protocols. The characterization and functional analysis of protein markers for somatic embryogenesis offer the possibility of determining the embryogenic potential of plant cells in culture long before any morphological changes have taken place, and of gaining further information on the molecular basis of induction and differentiation of plant cells.

The present review aims to summarize recent work that employs a variety of experimental approaches for the identification and use of protein markers for somatic embryogenesis in different species. The role of extracellular proteins as markers for somatic embryogenesis is especially emphasized.

1 Introduction

Somatic embryogenesis is a remarkable biological phenomenon. It is an ideal system for investigating the entire process of differentiation in plants, as well as of the mechanisms of expression of totipotency in plant cells. The three steps of embryogenesis from somatic cells, which comprise (a) induction of cell division, (b) induction of embryogenic potential, and (c) expression of the embryogenic program, include reprogramming of the gene expression pattern of the cells. The molecular basis of this unique developmental pathway, particularly the transition of somatic cells into embryogenic ones, is still the least understood (for a review, see Fehér et al. 2003). Markers for somatic embryogenesis help to establish embryogenic potential in plant cells for obtaining reasonable regeneration frequencies and provide information on the molecular mechanisms of plant cell differentiation. Different experimental approaches have been applied to isolate and characterize markers for somatic embryogenesis. In most cases, the comparative analysis of the total protein patterns from embryogenic and nonembryogenic cells resulted in a large number of specific proteins, making it difficult to use them as

markers (Hahne et al. 1988; Hilbert et al. 1992). The observation that the extracellular proteins are indispensable for differentiation and morphogenesis of plant cells, as well as their limited number when compared to the whole protein pattern, makes them appropriate candidates as markers for somatic embryogenesis. Indeed, many extracellular protein markers for embryogenic potential have been described (Sterk et al. 1991; De Jong et al. 1992; Kreuger and Van Holst 1993; Egertsdotter and Von Arnold 1995; Domon et al. 2000). Monoclonal antibodies against marker proteins have been useful in elucidating the complex structure of the plant cell surface, as well as for marking cells destined to develop somatic embryos (Toonen et al. 1996; Knox 1997; McCabe et al. 1997).

Differential cDNA screening has been widely applied to identify and characterize embryogenic markers (Schmidt et al. 1997; McCabe et al. 1997; Chugh and Khurana 2002). Differential display has been successfully used to isolate low-abundant genes (Alexandrova and Conger 2002; Yamazaki and Saito 2002; Charbit et al. 2004).

In this review, data are presented on the identification and use of early markers for somatic embryogenesis in different species by applying various experimental approaches.

2
Comparative Analysis of Proteins

2.1
Comparison of Protein Patterns after One- and/or Two-Dimensional Gel Electrophoresis

Biochemical aspects of the induction phase of somatic embryogenesis have so far been investigated at the protein level in many species. The first studies on carrot were reported by Sung and Okimoto (1981) who evidenced two 77- and 43-kD embryo-specific proteins. Similar studies performed on rice revealed the presence of several polypeptides in the range of 40 to 44 kD, which were more abundant in embryogenic calli than in nonembryogenic calli (Chen and Luthe 1987). The detection of embryogenesis-related proteins from total protein extracts has been reported for *Cichorium intybus* (Hilbert et al. 1992), *Dactylis glomerata* L. (Hahne et al. 1988), and *Cupressus sempervirens* (Sallandrouze et al. 1999). The analysis of total protein extracts from embryogenic versus nonembryogenic primary explants of the same origin allowed Pedroso et al. (1995a) to detect two polypeptides E_1 and E_2, specifically related to the process of proembryo induction and globular embryo development of *Camelia japonica*. Fellers et al. (1997) identified two proteins with 43 kD/pI 7.6 and 27 kD/pI 8.2 that can be used as markers for embryogenic potential in wheat callus. Blanco et al. (1997) found a marker protein

for the regeneration potential of sugarcane embryogenic callus. Hvoslef-Eide and Corke (1997) detected proteins specific for embryogenic cultures of birch. An investigation of total protein expression using two-dimensional gel electrophoresis during the ontogeny of carrot somatic embryogenesis enabled Dodeman and Ducreux (1996b) to identify markers of the induction phase and different developmental stages.

2.2 Comparison of Isoenzyme Patterns

The development of cells into embryogenic cell clusters and afterward into somatic embryos is accompanied by specific changes in protein pattern: new proteins are synthesized, others decrease and disappear. Changes in isozyme patterns have proved to be an efficient tool for analyzing the different stages in somatic embryogenesis. Isozyme expression is part of the controlled functional program involved both in acquisition of embryogenic potency and in the subsequent differentiation of the embryo. It has been shown earlier that isozyme responses vary with tissue organization during development and differentiation. Coppens and Dewitte (1990) found the esterase system to be very sensitive for the detection of embryogenesis in barley callus before somatic embryos are formed. In carrot, Chibbar et al. (1988) were able to detect two esterase isoenzyme systems differentially expressed in embryogenic and nonembryogenic cells. Esterase and peroxidase were found to be appropriate to discriminate between embryogenic and nonembryogenic callus in sweet potato (Cavalcante et al. 1994). Bapat et al. (1992) found several enzyme isoforms that discriminate between wheat embryogenic calli with regeneration potential and nonembryogenic calli that remain unorganized. A comparative analysis of ten somatic embryogenesis stages of carrot using a seven-enzyme system did not evidence any somatic embryogenesis-specific isozyme (Dodeman and Ducreux 1996a). Still other data indicate the potential of some enzymes to function as stage-specific markers for somatic embryogenesis. According to Bagnoli et al. (1998), the antioxidant enzymes superoxide dismutase and catalase could be convenient markers for defining the developmental stages in *Aesculus hippocastanum* somatic and zygotic embryos. The same role was postulated for peroxidase, whose isoenzyme patterns were shown to reflect the embryogenic potential of *Medicago sativa* (Hrubcová et al. 1994). The analysis of the electrophoretic patterns of specific enzymes proved to be an effective approach to the characterization of the main steps of *Vitis rupestris* somatic embryogenesis (Martinelli et al. 1993).

3
Antibodies Against Marker Proteins

3.1
Monoclonal Antibodies

Somatic embryogenesis involves a set of molecular events, both differential gene expression and various signal transduction pathways, for activating and/or repressing numerous sets of genes (Chugh and Khurana 2002). Studies on gene expression have revealed that embryo-specific genes are low-abundant genes and difficult to isolate. Differential hybridoma screening for the selection of monoclonal antibodies against marker proteins for somatic embryogenesis is more sensitive than two-dimensional gel electrophoresis, giving a chance of detecting low-abundant proteins. Antibodies are produced which may be used to monitor marker protein expression in different tissues and species. Smith et al. (1988) described a monoclonal antibody designated 21D7 that reacted with a nuclear protein associated with cell division in carrot somatic embryogenesis. Fukuda et al. (1994) proved that the 21D7 protein could be a candidate as an early marker of totipotency when cells start to divide and a competent cell becomes an embryogenic one. Kiyosue et al. (1990) generated a monoclonal antibody 1D11 against a 31-kD glycoprotein expressed in embryogenic cells but not in somatic embryos or nonembryogenic cells, and proposed that it should be a useful marker of embryogenic competence. Altherr et al. (1993) selected a monoclonal antibody 7C5 directed against a putative non-histone protein in *Pisum sativum* L. The acidic 50-kD protein was detected in other species, both dicots and monocots, and could serve as a marker for embryogenic potential. Monoclonal antibodies have been selected against germins (Lane et al. 1993). These proteins are associated with the cell wall and are one of the best-characterized markers for somatic embryogenesis in cereals.

The surface of plant cells includes the outer side of the plasma membrane, cell wall, middle lamella, and intercellular spaces. The monoclonal antibodies prepared against different components of the plant cell wall and extracellular proteins from the culture medium are useful molecular probes for studying the complex organization and dynamics of interaction between single components of the cell wall as a part of the plant extracellular matrix (Knox 1997, 1999; Smallwood et al. 1995, 1996; Willats et al. 2000). Arabinogalactan proteins (AGPs) are a class of proteoglycans implicated in diverse processes of plant growth and development, including somatic embryogenesis (for a review, see Showalter 2001). Presumably, AGPs are involved in molecular interactions and cellular signaling at the cell surface. Several antibodies have been prepared against diverse AGPs and were used to mark specific cell types (for reviews, see Knox 1997; Willats et al. 2000). A JIM4 antibody recognizing AGP epitopes in the protoderm of proembryogenic

masses (PEMs) and the culture medium of *Daucus carota* suspension cultures has been described (Stacey et al. 1990). Immunofluorescence using monoclonal antibody JIM4 has shown that the extracellular matrix surface network that covers the surface of embryogenic cells in friable maize callus is equipped with JIM4 epitope, while nonembryogenic callus cells are devoid of this epitope. Thus, JIM4 antibody can serve as an early marker of embryogenic competence in maize callus cultures (Samaj et al. 1999). The epitope of monoclonal antibody JIM13 is localized in epidermal cells (Knox et al. 1991), and Filonova et al. (2000) used it to distinguish PEMs from somatic embryos in *Picea abies*. JIM16 antibody recognized AGPs localized in the cell wall of peripheral cells of globular embryos and the culture medium and can be used as a marker for somatic embryogenesis in *Cichorium* (Chapman et al. 2000). ZUM18 recognizes AGPs with stimulatory effect on somatic embryogenesis in carrot (Kreuger and Van Holst 1995). Tchorbadjieva et al. (1998) isolated a monoclonal antibody 1D1, which recognizes two extracellular proteins from *D. glomerata* L. suspension cultures. The monoclonal antibodies against a range of polysaccharides and proteoglycan epitopes have been very useful in providing markers of developmental state and developmental potential. They have also helped to provide insight into aspects of cell-derived developmental signals (McCabe et al. 1997; Pennell 1998).

3.2
Phage Display Antibodies

Antibody technology has advanced in line with the development of molecular biological techniques. With the advent of phage display antibody technology there has been an extension of cell-based methods of generating monoclonal antibodies to gene-based methods (Winter et al. 1994). Phage antibody production is rapid and requires only very small amounts of antigen compared to hybridoma technology (Willats et al. 2000). A phage display monoclonal antibody PAM1 with specificity for de-esterified blocks of pectic homogalacturonan (HG) has been described (Willats et al. 1999a). In an intact cluster of suspension-cultured cells of *Arabidopsis thaliana* the PAM1 epitope is restricted to regions of cell-to-cell adhesion at the cell wall surface. A phage display antibody against the pectic component rhamnogalacturonan (RG) II has been isolated (Williams et al. 1996). Using a phage display subtraction method, Shinohara et al. (2000) were able to isolate monoclonal antibodies recognizing vascular development-specific cell wall components from *Zinnia* differentiating cells.

In conclusion, using both techniques, generation of monoclonal antibodies and phage display antibodies against components of the plant cell surface will provide further useful probes for studying the cell wall complexity and its structure–function relationships during somatic embryogenesis.

4
cDNA Differential Screening and Differential Display

4.1
cDNA Differential Screening

Many genes with altered expression during somatic embryogenesis have been identified; however, most of these are in late developmental stages (for reviews, see Chugh and Khurana 2002; Fehér et al. 2003). In the present review, only those experiments that aimed to isolate genes activated in the early stages of induction of somatic embryogenesis, with emphasis on their use as markers, will be described. Several different genes that are induced during somatic embryogenesis and are putative molecular markers have been isolated, typically by differential screening of cDNA libraries. These include genes encoding late embryogenesis abundant (LEA) proteins. The ECP31 transcripts were preferentially localized in the peripheral cells of embryogenic cells, and the authors suppose that ECP31 protein participates in the induction and/or maintenance of embryogenic competence (Kiyosue et al. 1992). *Emb-1* accumulates in the stage of maturation of somatic embryos (Wurtele et al. 1993). A cDNA clone for germin-like proteins (*PcGER1*) has been isolated whose transcripts are abundant in all embryogenic lines and absent from nonembryogenic lines of pine (Neutelings et al. 1998). They are localized in the walls of preglobular embryos and are markers for this early developmental stage. The approaches to identify genes activated during the early phases of chicory embryogenesis resulted in the identification of cDNAs of a β-1,3-glucanase (Helleboid et al. 1998).

The processes that govern the property of embryogenic competence in plant cells remain largely unknown (Mordhorst et al. 1997; Fehér et al. 2003). At present, there is only one gene known to play a role in the acquisition of embryogenic competence in plant cells. This is the somatic embryogenesis receptor kinase (*SERK*) gene (Schmidt et al. 1997). In carrot, *SERK* expression was shown to be characteristic of embryogenic cell cultures and somatic embryos whose expression ceased after the globular stage. Cell tracking experiments showed that *SERK*-expressing single cells could develop into somatic embryos; thus, *SERK* is considered to mark cells competent to form embryos in cell culture. The *Arabidopsis* homologue of the carrot *SERK* cDNA has also been cloned, and it was shown that the *AtSERK1* gene is highly expressed during embryogenic cell formation in culture and during early embryogenesis (Hecht et al. 2001). It was also established that the *AtSERK1* product is sufficient to confer embryogenic competence in culture. A carrot *SERK* homologue was shown to exist in embryos of *D. glomerata* L., and this gene can be used as a convenient marker to monitor embryogenic cell formation in monocots (Somleva et al. 2000). A *SERK* gene from *Medicago truncatula* (*MtSERK1*) has been isolated, orthologous to *AtSERK1*, which in legumes may

have a broader role in morphogenesis in cultured tissue rather than being specific for somatic embryogenesis (Nolan et al. 2003).

4.2 Differential Display

Genes involved in early stages of somatic embryogenesis have very low expression (Heck et al. 1995). Therefore, an alternative cloning method was developed in place of differential screening or subtractive hybridization. The differential display (DD) was first reported by Liang and Pardee (1992). In the last ten years, DD has been actively applied for the isolation of various genes from plants (review, Yamazaki and Saito 2002). It also turned out to be very effective in the isolation of genes involved in very early stages of somatic embryogenesis (Yoshida et al. 1994; Momiyama et al. 1995; Linkiewicz et al. 2004). Alexandrova and Conger (2002) identified two somatic embryogenesis-related genes DGE1 and DGE2 that were expressed in embryogenic but not in nonembryogenic leaf cultures from *D. glomerata* L. with possible nuclear regulatory functions. Charbit et al. (2004) isolated five cDNAs that could be used to distinguish between calli prior to induction, thus enabling an early diagnosis of callus embryogenic potential. Transcripts unique to embryogenic cell clusters in *Coffea arabica* (Rojas-Herrera et al. 2002), in cell clusters at the earliest stages of carrot somatic embryogenesis (Yasuda et al. 2001), and in embryogenic calli of *Lycium barbarum* (Kairong et al. 1999) have been detected.

5 Extracellular Proteins as Markers for Somatic Embryogenesis

The molecular basis of the unique developmental pathway of somatic embryogenesis, particularly the transition of somatic cells into embryogenic ones, is still the least understood (for review, see Fehér et al. 2003). Somatic embryogenesis in cell suspension cultures provides an alternative way to address this problem. The growth medium of plant cell cultures may be regarded as a large extension of the intercellular space; soluble secreted molecules that inhabit the apoplast *in planta* will accumulate in the medium when cells are grown in suspension. Thus, the complex array of molecules mainly derived from cell walls reflects the growth and development of cultured cells (Mordhorst et al. 1997). This opens up the possibility of studying the role of these molecules in early plant development, as well as searching for early markers for somatic embryogenesis among the secreted molecules. Suspension cultures secrete into the medium glycoproteins that play an important role in somatic embryogenesis by their ability to stimulate (De Vries et al. 1988; Kreuger and Van Holst 1993; Toonen et al. 1997a; Egertsdotter and

Von Arnold 1998; Domon et al. 2000) or inhibit (Gavish et al. 1992; Maës et al. 1997) somatic embryo development.

Comparison of extracellular protein patterns after one-dimensional or two-dimensional (2-D) gel electrophoresis showed that some proteins specifically appeared in embryogenic but not in nonembryogenic cell lines (De Vries et al. 1988; Nielsen and Hansen 1992; Tchorbadjieva et al. 1992; Kreuger and Van Holst 1993). Besides, it has been shown that suspension cultures of *Digitalis lanata* (Reinbothe et al. 1992) and *Dactylis glomerata* L. (Tchorbadjieva et al. 2004) differentiating into somatic embryos secreted proteins into the growth medium in a stage-specific manner. Analysis of extracellular proteins with the aid of 2-D protein gels was used to distinguish between different stages of somatic embryogenesis, and to identify putative candidates of proteins as markers for somatic embryogenesis (Tchorbadjieva et al. 2004). Some of these proteins were identified as an acidic esterase (Tchorbadjieva and Odjakova 2001), acidic lipid transfer protein-like proteins (Tchorbadjieva 2001), and an acidic endochitinase (Tchorbadjieva and Pantchev, 2006). All of these extracellular proteins were detected in a very early stage of somatic embryogenesis in *D. glomerata* L. embryogenic suspension cultures only, and could be used as early markers of embryogenic potential. Esqueda et al. (1998) identified two 34- and 36-kD polypeptides present in embryogenic cell suspension and involved in embryogenic development of sugarcane. An extracellular protein (46 kD, pI 6.1) was found that correlated with the embryogenic capacity of *Hordeum vulgare* L. cell cultures (Stirn et al. 1995). Domon et al. (1995) identified three glycoproteins secreted from embryogenic cell cultures of pine as germin-like proteins, one of the best-characterized markers of cereal embryo development (Lane et al. 1993). It was shown that during somatic embryogenesis of *Cichorium*, the change of the protein pattern in the medium is associated with the induction and initiation of somatic embryogenesis (Hilbert et al. 1992; Helleboid et al. 1995). Mo et al. (1996) observed that the morphology of somatic embryos of *Picea abies*, and especially that of the embryogenic regions, correlated with the presence of specific extracellular proteins that could be used to distinguish between normally developing embryos and embryos blocked in their development.

A first characterization of embryogenic suspension cultures, with respect to secreted esterases at defined stages of *D. glomerata* L. somatic embryogenesis, identified a unique acidic esterase that could discriminate on a biochemical level between *D. glomerata* L. embryogenic suspension cultures that regenerate whole plants and nonembryogenic suspension cultures (Tchorbadjieva and Odjakova 2001). Extracellular proteins secreted by distinct cell structures from embryogenic and nonembryogenic suspension cultures originating from the same genotype were submitted to isoelectric focusing (IEF) and stained for esterase activity (Fig. 1a). A new esterase A1 (pI 3.8) appeared in the phase when PEMs form from microclusters (Fig. 1a, lane 2). This isoenzyme persisted throughout the next phases until mature embryos developed

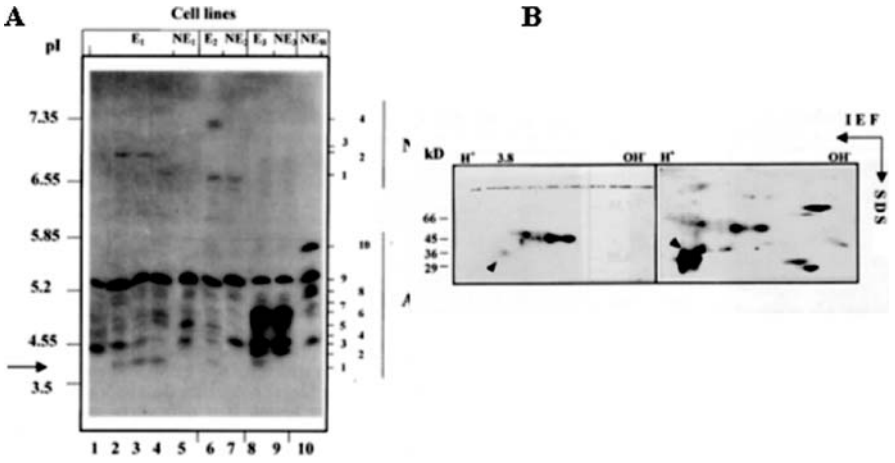

Fig. 1 Isoenzyme pattern of esterase activity of *Dactylis glomerata* L. suspension cultures after isoelectric focusing (**a**) and renaturation of esterases after two-dimensional gel electrophoresis (**b**). **a** Extracellular proteins harvested from the medium of: single cells → microclusters (*lane 1*); microclusters → PEMs (*lane 2*); PEMs → embryos (*lane 3*); embryos (*lane 4*) of E_1 embryogenic suspension culture; microclusters → PEMs (*lanes 6* and *8*) of E_2 and E_3 embryogenic suspension cultures; microclusters from NE_1, NE_2, NE_3, NE_W (*lanes 5, 7, 9, 10*, respectively) nonembryogenic suspension cultures. *Numbers on the right* refer to the position of the various isoforms of esterase activities of the A and N groups. Equal amounts of protein (7 μg) were loaded on each lane. The acidic esterase A1 (pI 3.8) is marked with an *arrow*. **b** Renaturation of extracellular esterases secreted by PEMs from E_1 embryogenic suspension culture in two-dimensional gel; *left panel*, slab gel stained for esterase activity only; *right panel*, the same gel subsequently silver-stained for protein. Molecular weight markers are as shown. The 36-kD esterase A1 is marked with an *arrow*

(Fig. 1a, lanes 3, 4). Among all esterase isoforms, only the presence of A1 was common to all embryogenic suspension cultures (Fig. 1a, lanes 4, 6, 8). In the nonembryogenic control lines (NE_1, NE_2, NE_3, NE_W) this enzyme was virtually absent (Fig. 1a, lanes 7, 9, 10). After 2-D SDS-PAGE electrophoresis and a successful renaturation, A1 occurred as a single polypeptide with an apparent molecular mass of 36 kD and pI 3.8 (Fig. 1b). Silver staining of the same gel showed it to be a moderately abundant protein (Fig. 1b). This unique esterase would allow for the identification of embryogenic potential at early stages of development before morphological changes have taken place.

One of the secreted proteins shown to play a key role in carrot somatic embryogenesis was identified as a 10-kDa lipid transfer protein designated EP2 (Sterk et al. 1991). It was found to be secreted only by embryogenic cells and somatic embryos as well as zygotic embryos. Studies revealed that expression was restricted to peripheral cells of proembryogenic masses (PEMs) and to protoderm cells of somatic embryos.

Nonspecific lipid transfer proteins (ns-LTPs) represent a protein family that is ubiquitous in plants (Kader 1996). These proteins are characterized

by their ability to transfer phospholipids between membranes and to bind fatty acids in vitro. Several in vivo functions have been attributed to ns-LTPs, including transport of cuticular compounds (Sterk et al. 1991) and inhibition of the growth of bacterial and fungal pathogens (Molina et al. 1993). Cutin is only present in embryogenic regions and on embryos as a homogeneous and continuous layer. One of the roles of a lipophilic substance like cutin in the cell wall of embryogenic cells is the physiological isolation of embryogenic competent cells from their neighbors as a prerequisite for organized development (Pedroso and Pais 1995b). The other role refers to the formation of a protective layer around the young embryo, which serves as protection against water loss, or the action of hydrolytic cell wall-degrading enzymes that are abundant in the conditioned medium. Expression of LTP gene is a well-known early marker of somatic embryogenesis induction in different systems (Sterk et al. 1991; Poulsen et al. 1996; Schmidt et al. 1997; Sabala et al. 2000). It is a marker for embryo differentiation as it is linked to the formation of the protoderm layer in developing somatic and zygotic embryos (Thoma et al. 1994). Furthermore, the *D. carota* EP2 is already expressed in precursor cell clusters from which somatic embryos develop. Taken together, a correct expression of *ltp* genes is required for normal embryo development. Five acidic LTP-like proteins have been found in the cell wall and the conditioned medium of microcluster cells from embryogenic suspension cultures of *D. glomerata* L. that could discriminate between embryogenic and nonembryogenic suspension cultures (Tchorbadjieva 2001).

One of the secreted proteins shown to have a positive effect on somatic embryogenesis in carrot was identified as a 32-kDa acidic endochitinase classified as a chitinase IV (De Jong et al. 1992). The endochitinase was able to rescue somatic embryogenesis in the mutant carrot cell line *ts*11. Chitinases (EC 3.2.1.14) catalyze the hydrolysis of β-1,4 linkages in chitin, a polymer of *N*-acetyl-D-glucosamine. Chitinases are expressed in many plant species in response to pathogen attack or to other environmental stresses (for a review, see Kasprzewska 2003). In the search for a plant-derived substrate for chitinase, Van Hengel et al. (2001) showed that AGPs from embryogenic suspension cultures contain *N*-acetyl-D-glucosamine and have cleavage sites for endochitinase. Pretreatment of AGPs with EP3 endochitinase resulted in optimal somatic embryo-forming activity. In addition to their putative role in plant defense responses, chitinases may also function in the development of somatic embryos, perhaps by releasing endogenous factors acting as signal molecules (Van Hengel et al. 2002). Chitinases released into the culture medium of *D. carota* (De Jong et al. 1992), as well as *Picea abies* (Mo et al. 1996) and *Pinus caribaea* (Domon et al. 2000) embryogenic cell lines, have been reported to influence somatic embryo development. In *D. glomerata* L. suspension cultures a 32-kD acidic endochitinase has been found to be expressed constitutively in embryogenic suspension cultures and during all stages of somatic embryogenesis (Tchorbadjieva and Pantchev 2006),

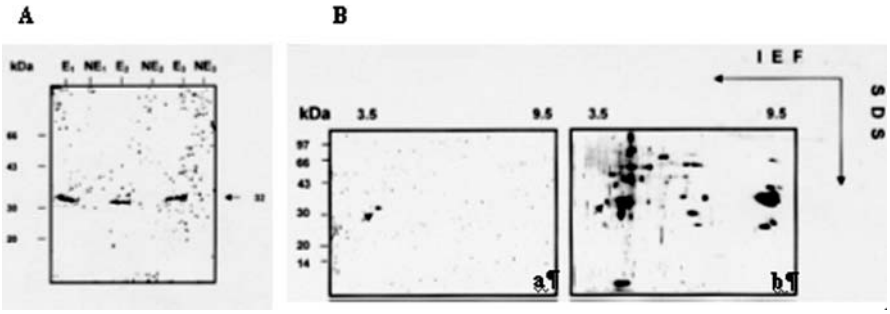

Fig. 2 Detection of a chitinase-like protein in culture media of *Dactylis glomerata* L. suspension cultures. **a** Immunoblot with extracellular proteins from embryogenic (E_1, E_2, E_3) and nonembryogenic (NE_1, NE_2, NE_3) suspension cultures with anti-32-kDa chitinase serum (De Jong et al. 1995). **b** Immunoreactivity of the extracellular proteins secreted by PEMs from E_3 embryogenic suspension culture with anti-32-kD serum from carrot after 2-D gel electrophoresis; *panel a*, immunoblot; *panel b*, silver-stained duplicate gel. The 32-kD acidic chitinase-like protein (pI 3.6) is shown with an *arrow*. Molecular mass markers are shown on the *left*

and could serve possibly as a marker for embryogenic potential (Fig. 2a). Two-dimensional gel electrophoresis and immunoblotting with anti-chitinase antiserum showed that the band of 32 kDa obtained after 1-D separation of E_3 extracts resolved in a unique spot located in the acidic part of the electrophoretogram (Fig. 2b, panel a). We assume that it could possibly serve as a marker for the embryogenic potential of *D. glomerata* L. suspension cultures. This is in agreement with the results of Mo et al. (1996), who found a correlation of chitinase secretion in a *Picea abies* in vitro culture with the ability of PEMs to form normal somatic embryos. Domon et al. (2000) reported the identification of a 48-kDa chitinase-like protein, ionically bound to the surfaces of preglobular somatic embryos of Caribbean pine. Two chitinase isoforms were shown to accumulate in the medium of embryo cultures to a much higher level compared to that in the medium of a nonembryogenic *Cichorium* variety (Helleboid et al. 2000). Wiweger et al. (2003) revealed that *Chia 4-Pa* chitinase genes were expressed in a subpopulation of proliferating cells and at the base of the somatic embryo in *Picea abies*, and that the protein promotes PEM-to-somatic embryo transition. Egertsdotter and Von Arnold (1998) observed a stimulating effect of a chitinase-4 related chitinase on early embryo development in Norway spruce suspension cultures.

Arabinogalactan proteins (AGPs) are proteoglycans commonly found in the cell wall, cell matrix, and cell membrane of plants. Different hypotheses propose that AGPs may be involved in cell proliferation, cell expansion, and regulation of somatic embryo development (for a review, see Showalter 2001). Promotive and inhibitory to somatic embryogenesis effects of certain exogenously added AGPs were reported for carrot cultures (Kreuger and Van Holst 1993; Toonen

et al. 1997a) and Norway spruce cultures (Egertsdotter and Von Arnold 1998). In *Cichorium*, immunofluorescence studies localized AGPs to the outer cell wall of globular somatic embryos, and they were abundantly present in the culture medium, too (Chapman et al. 2000). Several antibodies have been prepared against diverse AGPs and were used to mark specific cell types (for reviews, see Knox 1997; Willats et al. 2000). An AGP epitope from carrot cell-conditioned medium recognized by the JIM8 antibody was originally described as a marker of the very early transitional stage of cultured carrot cells after embryogenic induction (Pennell et al. 1992). Subsequently it was shown that most embryos develop from cells lacking the JIM8 epitope (Toonen et al. 1996). Finally, it was found that the JIM8 epitope marks a specific cell type that, upon cell division, asymmetrically transferred the JIM8 epitope to a $JIM8^-$ embryogenic and $JIM8^+$ apoptotic cell type. It was further demonstrated that the JIM8 epitope represents a soluble signal produced by $JIM8^+$ cells to stimulate embryo development of $JIM8^-$ cells (McCabe et al. 1997). We isolated a monoclonal antibody MAb 3G2 against a cell wall protein designated EP48 secreted by the earliest morphological structures (microclusters) in *D. glomerata* L. embryogenic suspension cultures (Tchorbadjieva et al., 2005) (Fig. 3a). Screening of

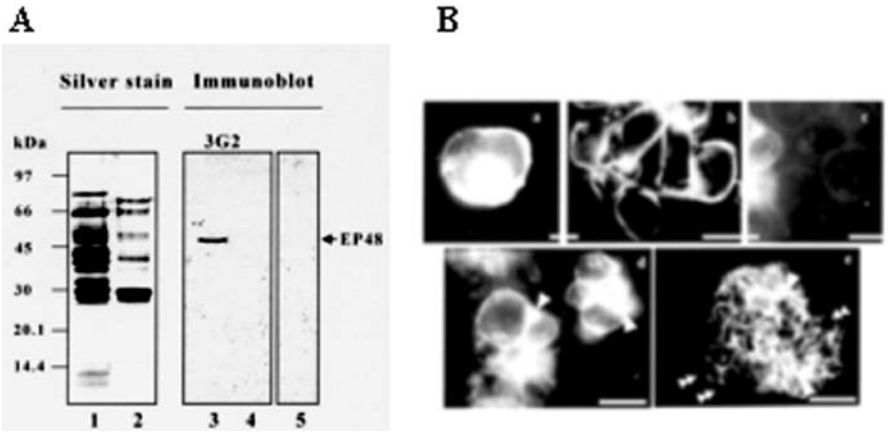

Fig. 3 Immunoblot analysis of extracellular proteins with monoclonal antibody MAb 3G2 (**a**) and indirect immunofluorescent localization of EP48 on intact *D. glomerata* L. suspension cells during somatic embryogenesis (**b**). **a** Immunoblot of extracellular proteins from embryogenic (*lanes 1* and *3*) and nonembryogenic (*lanes 2* and *4*) microcluster cells after SDS-PAGE and transfer to PVDF membrane. MAb 3G2 recognized a single protein (M_r 48 000) (*arrow*). The control with preimmune serum (*lane 5*) was negative. Molecular mass markers are indicated on the *left* in kD. **b** MAb 3G2 labeled the cell wall of small, isodiametric single cells (*a*) as well as elongated, banana-shaped single cells (*b*); many single cells (*c*) remain unstained. The fluorescence due to the antibody binding is most intense at the regions of cell adhesion of microcluster cells (*d*) and PEMs (*e*) (*single arrowheads*), while regions of cell wall without neighbors are unlabeled in PEMs (*double arrowheads*). Bars = 10 μm (a); 30 μm (b–e)

the extracellular proteins from microclusters of three embryogenic (E_1, E_2, and E_3) and nonembryogenic (NE_1, NE_2, and NE_3) suspension cultures on immunoblots showed that EP48 was found exclusively in the embryogenic cell lines. Immunofluorescence localized EP48 on the cell surface of some single cells, microclusters, and PEMs. Interestingly, in microclusters immunofluorescence was located at sites of cell–cell contact but could also be found on cell surface regions that were not in direct contact with neighboring cells, while in PEMs the distribution of EP48 was uneven, and was less intense or even absent from the regions of the surface of PEMs where cells had no neighbors (Fig. 3b). Possibly, during development of PEMs a local change in the cell wall of some cells occurred leading to the loss of MAb 3G2 epitope. Whether the monoclonal antibody marks cells destined for embryogenesis remains to be elucidated, but based on its localization and pattern of accumulation we conclude that it can be useful to monitor the embryogenic potential of *D. glomerata* L. suspension cultures.

It is now widely recognized that the extracellular proteins are indispensable for differentiation and morphogenesis, taking part in signal transduction, cell–cell recognition, cell expansion, and adhesion.

6 Conclusion

In the preceding section, protein markers for somatic embryogenesis and the different experimental approaches for their identification and use have been discussed. The protein markers are useful probes for defining embryogenic potential and for marking different phases in plant development. To gain a better insight into the mechanisms of somatic embryogenesis, a combination of more advanced methods such as the phage display subtraction method, differential display, and proteome analysis is indispensable. Immunomagnetic sorting and cell tracking could be successfully applied to determine the fate of embryogenic cells. All this will greatly accelerate the functional analysis of protein markers, and will contribute to the improvement of crop species together with the establishment of efficient propagation technologies.

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