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CYSTEINE PROTEINASES AND SOMATIC EMBRYOGENESIS IN SUSPENSION CULTURES OF ORCHARDGRASS (*DACTYLIS GLOMERATA* L.)

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Summary. Somatic embryogenesis in cell suspension cultures provides a good model system for investigating early plant development. The conditioned medium harbors a complex array of molecules, mainly derived from the cell walls, which may exert a promotive or inhibitory effect on embryo development. Cell wall proteome and secretome analysis of *Arabidopsis*, *Oryza sativa*, *Nicotiana tabacum*, *Medicago sativa* and *Vitis* showed the presence of an unexpected large number of proteases in the cell wall and the culture medium with putative signaling function. Sodium dodecyl sulphate (SDS) – polyacrylamide gel copolymerized with gelatin was used for one-dimensional (1D) and two-dimensional (2D) - electrophoresis to analyze the proteases from the medium of embryogenic suspension cultures of *Dactylis glomerata* L. during early stages of somatic embryogenesis and non-embryogenic suspension cultures during unorganized cell proliferation. A varying set of acidic protease isoenzymes accompanied the development of both lines. Based on the sensitivity to specific protease inhibitors, a 70 kDa serine proteinase and several 36 kDa cysteine proteinase isoforms (pI 4-5.5) were identified. The 70 kDa serine proteinase was found in the medium and the cell wall of the embryogenic and non-embryogenic cell lines during all stages of development. Interestingly, the 36 kDa cysteine proteinase isoforms were present in the extracellular proteins from the medium and the ionically bound cell wall fraction of the embryogenic cell lines only. We suggest that the enzyme(s) may play an essential role for early somatic embryo development.

Key words: extracellular proteins, orchardgrass *(Dactylis glomerata* L.), secreted cysteine proteinases, somatic embryogenesis, suspension culture.

Abbreviations: Dicamba - 3, 6-dichloro-o-anisic acid; SH0 - Schenk and Hildebrandt (1972) medium; SH30 - SH medium, supplemented with 30 μM dicamba; PMSF - phenylmethyl sulfonyl fluoride; E-64 - *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino) butane; EDTA – Ethylenediaminetetraacetic acid; EGTA - ethylene glycol-bis (2-aminoethylether)-N,N,N',N'-tetraacetic acid; IAA-iodoacetamide.

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INTRODUCTION

Somatic embryogenesis is a unique phenomenon in the plant kingdom. Somatic embryogenesis is the process by which somatic cells develop into plants through characteristic morphological stages. The transition of somatic cells into cells that are capable of forming an embryo is the most important and the part of somatic embryogenesis least understood (for review, see Karami et al., 2009). Somatic embryogenesis in cell suspension cultures provides a good model system for investigating early plant development. Conditioned medium of cell suspension cultures may be regarded as a large extension of the apoplast. It harbors a complex array of molecules, mainly derived from the cell walls, which may exert a promotive or inhibitory effect on embryo development (Matthys-Rochon, 2005).

Studies of somatic embryogenesis show that asymmetric cell division and controlled cell expansion are important mechanisms for the generation of embryogenic plant cells indicating a major role for the plant cell wall in these processes (Fehér et al., 2003). The plant cell wall is a highly dynamic structure whose structure and composition changes dramatically during cellular differentiation. Regulation of the cell wall properties may be achieved by addition of new cell wall material and/or modification of existing components by cell wall enzymes (Jamet et al., 2008). Results from proteomics and secretomics have revealed the existence of a large number of soluble secretory proteins, including a great diversity of proteases, such as cysteine- and aspartyl proteases, subtilases and carboxypeptidases (Jamet et al., 2008; Agrawal et al., 2010). Proteases are classified based on their catalytic mechanisms into cysteine proteases, serine proteases, metalloproteases, threonine proteases, aspartic proteases and glutamic acid proteases. Besides their well-known function to degrade nonfunctional proteins, proteases certainly play key roles in the maturation of cell wall proteins and in the generation of active peptides in the cell wall thus regulating different processes in response to developmental and environmental cues (for review, see van der Hoorn, 2008). Proteases can contribute to plant embryogenesis, too. A calpain-like membrane protease Defective kernel 1(DEK1) is required for epidermal cell identity, which is essential for the development of the embryo and the suspensor (Johnson et al., 2005). Abnormal leaf epidermis 1 (ALE1) is responsible for cuticle development during embryogenesis. The *ALE1* gene encodes a S8 subtilisin-like serine protease. It is suggested that it is secreted by the endosperm and promotes cuticle formation on the embryo (Tanaka et al., 2001). Mitsuhashi et al. (2004) found at least four cysteine proteases in carrot somatic embryos, which changed dynamically during somatic embryogenesis depending upon the developmental stages. Tian et al. (2009) identified a cysteine proteinase (OsCP) involved in proliferation of suspension-cultured rice cells. The biological significance of protein cleavage by proteases in the cell wall, the precise mechanism of their action and the control for this action, as well as their possible role during somatic embryogenesis

remains largely unknown. In the present study, we used an activity-staining method to identify specific proteases that contribute to the development of *D. glomerata* L. embryogenic suspension cultures. Several acidic 36 kDa cysteine proteinase isoforms (pI 4-5.5) were found in the medium of embryogenic cell lines only. Research is currently underway to determine their structure and function in early somatic embryogenesis.

MATERIALS AND METHODS

Plant material and suspension cultures

Callus-derived suspension cultures from two embryogenic (E1, E2) and two non-embryogenic (NE1, NE2) cell lines of orchardgrass (*Dactylis glomerata* L.) were initiated according to Conger et al. (1989) and maintained in a liquid SH30 medium essentially as previously described (Tchorbadjieva and Odjakova, 2001).

Protein preparation

The culture medium from suspension-cultured cells was collected every 3 days after subculturing until 20 days later and cleared by filtration through a Millipore 0.45 μm filter. Proteins secreted during embryogenesis were extracted from the medium as described by De Vries et al. (1988). Briefly, proteins were precipitated by the addition of 2.5 volumes of ethanol and after overnight at 4ºC the precipitate was collected by centrifugation (12000xg at 4ºC for 30 min), vacuum-dried and stored at -70ºC or dissolved in water for immediate use. Cell wall proteins were prepared from washed cells after membrane filtration by sequential extraction with 0.2 M $CaCl_2$ and 1 M NaCl. Extracts were dialyzed against a 10-fold excess of dH_2O, lyophilized and reconstituted in ddH_2O (Robertson et al., 1997). Intracellular soluble proteins were obtained by grinding the cells in extraction buffer containing 50 mM Tris–HCl, pH 7.5, 1 mM EDTA and 5 mM $MgCl_2$. The homogenate was filtered through nylon gauze, centrifuged at 15,000 g at 4°C for 30 min and the supernatant was recovered (Tchorbadjieva et al., 2005). The protein content was determined according to Bradford (1976).

Gel electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Okadjima et al. (1993). Analytical flat bed isoelectric focusing (IEF) of extracellular proteins was carried out on 5% (w/v) polyacrylamide gels containing ampholines in the pH range of 3.0 to 9.5 (GE Healthcare). After IEF the excised lanes were separated by SDS-PAGE in the second dimension. Proteins were stained with silver nitrate according to Blum et al. (1987). For detection of protease activity on 2D gels, the second dimension SDS-PAA gel contained 0.1% gelatin and was further developed as described in the section ”Determination of “*in gel*” protease activity”.

Determination of “*in gel*” protease activity

Detection of protease activity was done using non-boiled samples in 13% acrylamide gels containing 0.1% gelatin. After renaturation in 2.5% Triton X-100

solution at room temperature for 30 min, the gel was rinsed with distilled water and developed overnight at 37˚C in proteolysis buffer (100 mM citrate phosphate buffer, pH 6.0; 10 mM L-cysteine). Staining with 0.05% Coomassie Brilliant Blue R-250 in 10% acetic acid - 25% isopropanol followed and areas of protease activity were identified as clear bands against a blue background (Azzez et al., 2007). To identify the class of proteases observed on the gels, protein samples were incubated with different protease inhibitors (100 μM E-64, 1 mM PMSF, 10 mM IAA or 10 mM $ZnSO_4$) before addition of the loading buffer.

RESULTS

Suspension cultured cells from the *D. glomerata* L. embryogenic cell lines (E1, E2) reached the log phase between 6 and 9 day after subculturing (Tchorbadjieva and Odjakova, 2001). At day 3 after subculturing microcluster cells divide to form proembryogenic masses (PEMs) and at day 6 after subculturing PEMs give rise to early globular embryos which develop into mature embryos during days 9-12 after subculturing. When put on a thin layer of liquid SH-0 medium, mature embryos readily germinated and produced plantlets. Single cells from the non-embryogenic cell lines (NE1, NE2) divided to form microclusters, whose further development was blocked. The extracellular proteins from the medium of 6 day embryogenic cell cultures (EEPs) were isolated and compared with those in the cell walls and the cytosol on SDS-PAGE gel electrophoresis (Fig. 1). For the extraction of ionically bound cell wall proteins (CWPs) we employed a non-destructive sequential extraction

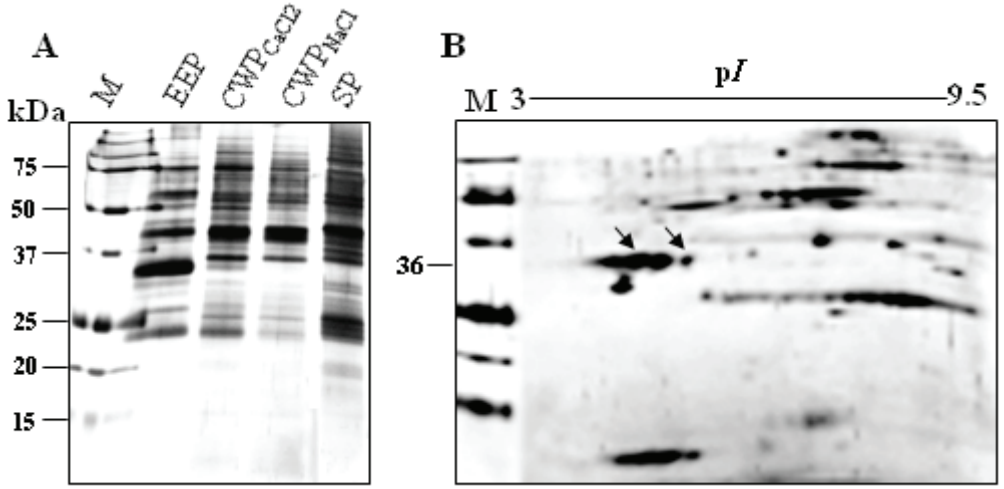

Fig. 1. Analysis of electrophoretic patterns in *D. glomerata* L. E1 embryogenic suspension culture at day 6 after subculturing. Proteins from the medium (EEP), ionically bound cell wall proteins after sequential extraction with $CaCl_2$ ($CWP_{CaCl2)}$ and NaCl (CWP_{NaCl}) and soluble intracellular proteins (SP) were separated on 13% SDS-PAGE (A). 2D-PAGE pattern of extracellular proteins. Arrows show the two cysteine proteinases identified by MS analysis (B). The gels were silver stained. M, molecular mass markers.

with $CaCl_2$ and NaCl from intact suspension cultured cells thus avoiding contamination with intracellular proteins. The results show that the proteins from the culture medium display a different profile from that of their respective cell wall fractions. Similarly, there are distinct subsets of proteins extracted by $CaCl_2$ and NaCl. Besides, the intracellular protein pattern differs significantly. This, together with the very low activity of the cytoplasmic marker glucose-6-phosphate dehydrogenase determined in a previous study with the same suspension cultures (Tchorbadjieva and Odjakova, 2001) and the high viability of the suspension cells tracked by Evan's blue (data not shown) implies that the extracellular proteins are derived from the extracellular (or apoplastic) environment and are not due to cell leakage. Two-dimensional (2D) gel electrophoresis of the extracellular proteins showed the presence of ca. 40 polypeptides with a wide range of molecular masses (10-120 kDa) and pIs (3.5-9.5). A partial secretome analysis of the secreted proteins identified 14 proteins, among them cysteine proteinase and cathepsin B (Fig. 1B, arrows) (manuscript in preparation). This result prompted us to screen the culture medium for protease activity. For the purpose, we used an in-gel activity staining method to detect the isoenzyme profile. A set of three broad activity bands with molecular mass of 30-36 kDa, 70-80 kDa and 200 kDa among the extracellular proteins secreted into the medium of embryogenic suspension cultures was detected (Fig. 2A). The protease activity pattern changed both qualitatively and quantitatively during development. The 200 kDa protease was constitutively expressed for all time periods tested and gradually diminished at later stages. A 75 kDa proteinase band appeared at day 6, its activity remained constant during the next stages of development and only slightly increased at day 20. The most substantial changes in the protease activity were observed in the 30-36 kDa activity bands (Fig. 2A). The 36 kDa protease activity appeared in the most early stages of development (days 3 and 6), followed by two 30 kDa and 32 kDa activities (days 9 and 15) and finally, all three 30-36 kDa activities marked the latest stage when conditioned suspension cultures were established. To check our assumption that the proteases present in the medium are derived from the apoplastic environment, we then compared the extracellular protease pattern and the corresponding cell wall protease pattern (Fig. 2B). The 200 kDa activity was present in both $CaCl_2$ - and NaCl- extracted cell wall proteins. The other two broad bands of 70-80 kDa and 36 kDa activities were present, too, though with much lower activity. The soluble intracellular proteins contained both the 200 kDa and 70-80 kDa protease activities with no 36 kDa activity been observed. The protease activity pattern of the extracellular proteins from the non-embryogenic cell lines differed from that of the embryogenic cell lines: in NE1 line, only the 200 kDa activity was observed and in NE2 the 70-80 kDa activity was present. The most remarkable difference was the absence of the 36 kDa activity in both non-embryogenic cell lines. The 2D gel electrophoresis of the extracellular proteases from the E1 embryogenic cell line showed that all protease enzymes were acidic ones, with pIs in the range 4-5.5 (Fig. 2C). It is evident from the 2D

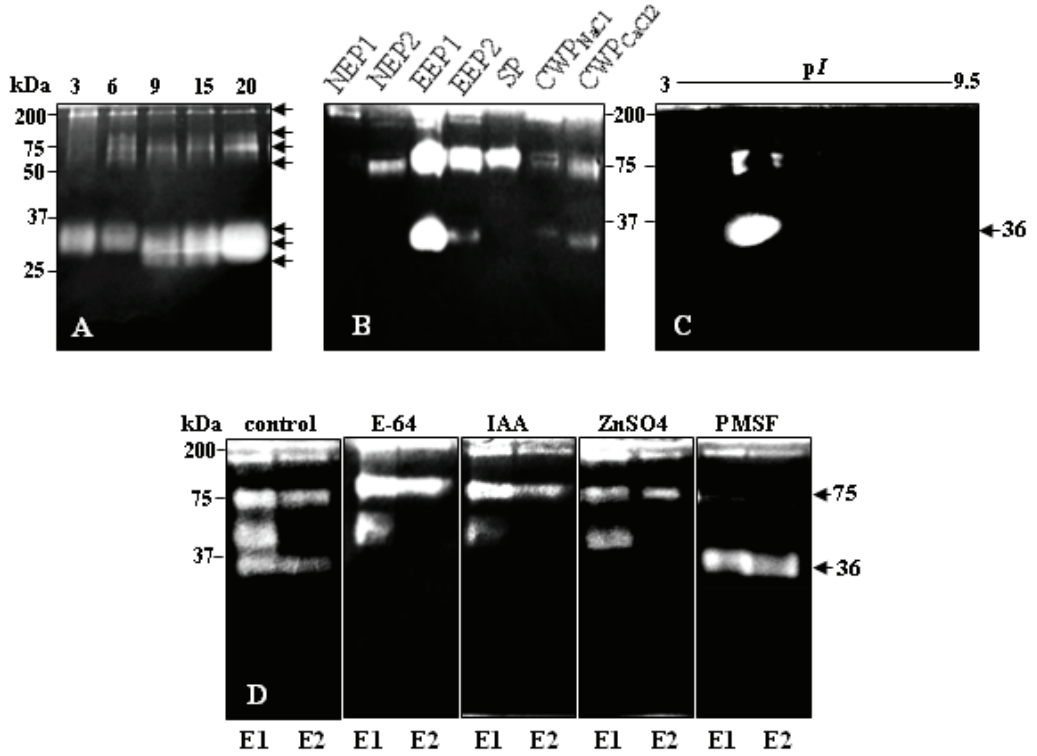

Fig. 2. Gelatin zymogram 1D and 2D of *D. glomerata* L. suspension culture proteases. *D. glomerata* L. extracellular proteins EEP1 isolated from the culture medium at the indicated days after subculturing (numbers above) (A) and extracellular proteins from non-embryogenic cell lines (NEP1, NEP2), embryogenic cell lines (EEP1, EEP2), cell wall proteins recovered by sequential extraction with $CaCl_2$ (CWP_{CaCl_2}) and NaCl (CWP_{NaCl}) and soluble intracellular proteins (SP) were analyzed by 13% SDS-PAGE in a gel containing 0.1% gelatin and submitted to zymography (B) (see Materials and Methods).
2D zymogram: *D. glomerata* L. extracellular proteins at day 6 after subculturing were resolved in the first dimension on a flat bed isofocusing gel (3–9.5) and in the second dimension by 13% SDS-PAGE gel, containing 0.1% gelatin (C). Effects of proteinase inhibitors on the activity of extracellular proteinases of E1 and E2 embryogenic cell lines (D). The 13% SDS-PAGE gel was copolymerized with 0.1% gelatin. On the right side are shown the 70 kDa and 36 kDa proteinases, specifically inhibited by PMSF and E-64, $ZnSO_4$ and IAA, respectively.

gel that the 36 kDa protease separated into more than one protein, but the activity spots were very diffuse and we could not determine the precise number of the protein isoforms. The three broad activity bands were classified using the class-specific protease inhibitors: E-64, iodoacetamide and $ZnSO_4$ for cysteine proteases and PMSF for serine proteases (Fig. 2D). The 200 kDa protease activity was not inhibited by any of the inhibitors used. PMSF inhibited strongly the activity of the 75 kDa band suggesting that this is a serine proteinase. The activity of the 36 kDa band was drastically inhibited by treatment with E-64, IAA and $ZnSO_4$, but

not by treatment with PMSF. The results strongly suggest that the 36 kDa isoforms are cysteine proteinases. Treatment with the metalloproteinase-specific inhibitors EDTA/EGTA exerted no effect on any of the observed activities (data not shown).

DISCUSSION

Analyzing suspension culture medium offers an easy and convenient way to study freely soluble secreted proteins and cell wall proteins with little contamination from intracellular proteins (Kusumawati et al., 2008). In the present study, we compared the protein pattern of extracellular proteins secreted by *D. glomerata* L. embryogenic suspension cultures with that of their corresponding cell wall proteins. The majority of extracellular proteins were absent from their respective cell wall extracts, which points to a certain speciation in the complement of extracellular and cell wall proteins. The different pattern of the intracellular proteins and the high viability of the cells in the suspension cultures pointed to a negligible contamination with intracellular proteins. Robertson et al. (1997) observed similar results when comparing the extracellular proteins and the cell wall proteins of five different plant species. Most of the protein sequences found within the culture filtrates were absent from the subsequent salt-eluted extracts. Our attempt to screen the extracellular proteins secreted by the embryogenic suspension cell cultures during defined stages of development for protease activity revealed the presence of three broad zones of digested gelatin, containing a range of proteases with molecular masses of 30-36 kDa, 70-80 kDa and 200 kDa (Fig. 2A). We observed dynamic changes in the activity bands accompanying the developmental stages. For example, a 36 kDa proteinase marked the most early stages of development, appearing at days 3 and 6 and finally, on day 20. The three enzymes consisting the 30-36 kDa activity zone showed highest activity on day 20, which could be attributed to the starting of culture senescence. On day 3 and day 6 after subculturing substantial differences in the overall pattern of protease activity were observed, which might be correlated to regulation of critical steps in the early stages of somatic embryogenesis. All proteases detected were acidic proteins with pIs 4-5.5 (Fig. 2C).

Proteases might play an important role in somatic embryogenesis. During carrot somatic embryogenesis, active protease bands and cysteine proteinase gene expression were reported and changed in relation to developmental stages (Mitsuhashi et al., 2004). Parisi et al. (2002) observed a cysteine proteinase secreted by garlic callus in the early stages of development. Analysis of the cell wall proteases showed the same pattern as that of the extracellular ones though with much weaker activity (Fig. 2B). Calcium chloride has been shown to effectively extract many wall-bound proteins and NaCl - to liberate proteins that were strongly ionically associated with the wall. The in-gel activity staining indicates that orchardgrass cell culture medium contains a substantial quantity of 36 kDa and 75 kDa proteases. Both enzymes are present in the cell wall as weakly bound

proteins whose labile binding could explain their abundance in the medium. The 200 kDa and the 75 kDa proteases are common to both embryogenic and non-embryogenic cell lines, which points to their participation in common processes during development. However, the most striking fact that we observed was the presence of the 36 kDa proteinase in the medium and as a weakly bound protein in the cell wall of embryogenic suspension cultures only. No such activity was detected in the soluble fraction. As the induced suspension cultures of both embryogenic and non-embryogenic lines had the same explant origin and they were submitted to the same culture procedures, the 36 kDa proteinase detected only in the embryogenic lines was considered as embryogenesis-associated protein. Its absence from the medium of the non-embryogenic culture could be related to the blocked synthesis or inhibition of the enzyme in the process of loosing embryogenic potential. The early stages of development of the suspension cultures-days 3 and 6, are marked with a very intensive cell proliferation and differentiation. It is tempting to speculate that the 36 kDa cysteine proteinase takes part in a local modification of the cell wall or that it generates signals, necessary for somatic embryogenesis. The opposite action of a cysteine proteinase (OsCP) and a cystatin (OC-1) was found to control cell proliferation in rice suspension cultures (Tian et al., 2009). Kusumawati et al. (2008) identified three secreted proteases only in an embryogenic cell line of *Medicago truncatula*. Class-specific protease inhibitors were used to discriminate between the different proteases defining the 36 kDa proteases as cysteine proteinases and the 75 kDa protease as serine proteinase (Fig. 2D). The role that the proteases found in the medium of *D. glomerata* L. suspension cultures might play remains to be elucidated. The presence of extracellular proteases is of interest since they are likely candidates for apoplastic processing and are known to play important roles in plant development (Jamet et al., 2008). A mass spectroscopic analysis identified two proteins from the 36 kDa band as cysteine proteinase and cathepsin B (GenBank accession numbers GU067466.2 and GU067465.1, respectively). The full-length cDNAs for both cysteine proteinases were cloned and expressed (to be published elsewhere). Major biological roles for proteolytic activities were only recently demonstrated in maturation of enzymes or production of extracellular peptidic signals. The availability of recombinant cysteine proteinases in our laboratory will stimulate further research to find their targets in the medium and the cells, to determine their structure and to elucidate their role in somatic embryogenesis.

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