BIOLOGIA PLANTARUM 50 (1): 142-145, 2006

BRIEF COMMUNICATION

Secretion of a chitinase-like protein in embryogenic suspension cultures of *Dactylis glomerata* L.

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Abstract

A chitinase-like 32 kDa acidic protein with a potential chitinase activity has been identified in the medium of embryogenic suspension cultures of *Dactylis glomerata* L. using an antiserum raised against endochitinase EP3 from *Daucus carota* L. The presence of this protein discriminates between *Dactylis glomerata* L. embryogenic and non-embryogenic suspension cultures and thus could be possibly used as a marker for embryogenic potential.

Additional key words: extracellular proteins; orchardgrass; somatic embryogenesis.

Somatic embryogenesis is a good model system for studying the molecular aspects of early plant development. Several studies have demonstrated either a promotive (De Vries *et al*. 1988, Egertsdotter and Von Arnold 1995, Toonen *et al*. 1997, Domon *et al*. 2000) or inhibitory (Gavish *et al*. 1992, Maës *et al*. 1997) role of extracellular proteins from the medium of suspension cultures. One of the secreted proteins shown to rescue somatic embryogenesis in the mutant carrot cell line *ts*11 was identified as a 32 kDa acidic endochitinase (De Jong *et al*. 1992).

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 (Burketová *et al*. 2003/4, Kasprzewska 2003, Thangavelu *et al*. 2003). In search for a plant-derived substrate for chitinase Van Hengel *et al*. (2001) showed that arabinogalactan proteins (AGP) from embryogenic suspension cultures contain N-acetyl-D-glucosamine and have cleavage sites for endochitinase. In addition to their putative role in plant defence responses, chitinases may also function in the development of somatic embryos, perhaps by releasing endogenous factors acting as signal molecules.

Previously an acidic abundant 32 kDa protein was detected among a group of extracellular proteins that were found in all stages of *D. glomerata* L. somatic embryogenesis (Tchorbadjieva 2004). In the present study, we identified this protein as an acidic chitinase-like protein that is constitutively secreted in embryogenic suspension cultures only. We assume that the accumulation of this protein in the culture medium could be correlated with the process of somatic embryogenesis.

Callus-derived suspension cultures from three embryogenic (E_1, E_2, E_3) and three non-embryogenic (NE_1, NE_2, NE_3) 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).

Received 23 April 2004, *accepted* 7 September 2004.

Abbreviations: Dicamba - 3,6-dichloro-*o*-anisic acid; IEF - isoelectric focusing: PEMs - proembryogenic masses; pI - isoelectric point; SDS-PAGE - sodium dodecyl sulphate polyacrylamide gel electrophoresis; SH0 - Schenk and Hildebrandt (1972) medium; SH30 - SH0 medium supplemented with 30 μM dicamba; 2-D PAGE - two-dimensional polyacrylamide gel electrophoresis.

Acknowledgements: We wish to thank Dr. Sacco de Vries (Wageningen University, The Netherlands) for a generous gift of the polyclonal antibodies against endochitinase EP3.

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After 7 d in culture, the culture media of globular embryos, proembryogenic masses (PEMs) and microclusters from embryogenic suspension cultures were separated from the cells and the extracellular proteins were recovered by precipitation with ethanol (Tchorbadjieva and Odjakova 2001). The microclusters from the non-embryogenic cultures were maintained in the same manner. Protein determination followed the method of Bradford (1976) using BSA as a standard.

Extracellular proteins were separated by SDS-PAGE according to Okadjima *et al*. (1993) using a 13 % acrylamide separating gel and a 4 % acrylamide stacking gel. As molecular mass standards phosphorylase B (97 kDa), bovine albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa) were used.

Isoelectric focusing (IEF) was carried out on 0.75 mm thick acrylamide slab gels (5 %), using 2 % (v/v) ampholine carrier ampholytes, pH 3.5 to 9.5 (*Pharmacia Biotech*, Uppsala, Sweden). Proteins were stained with silver nitrate (Blum *et al*. 1987).

Polyclonal antiserum against endochitinase EP3 class IV from *D. carota* L. was a gift from Dr. Sacco De Vries (Wageningen University, Wageningen, The Netherlands). Upon SDS-PAGE or 2-D PAGE separation, the proteins were electrotransferred to PVDF membranes using semi-dry transferring system. The development of the membrane was performed essentially as described (Albiach *et al*. 1994).

Chitinolytic activity was detected in SDS-PAGE (with omission of β-mercaptoethanol) gels, containing 0.01 % (m/v) glycol chitin according to Marek *et al*. (1995).

As mentioned previously (Tchorbadjieva 2004), different stages of somatic embryogenesis in suspension cultures of *D. glomerata* L. have been characterized by specific profiles of their secreted proteins. 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. To identify chitinase-like proteins, Western blots after 1-D separation of extracellular proteins from the embryogenic and their corresponding non-embryogenic cell lines were probed with an antibody raised against carrot endochitinase EP3 (De Jong *et al*. 1995). The antibody cross-reacted with extracellular proteins from embryogenic cell lines only, recognizing a single band of 32 kDa (Fig. 1*A*). Since both embryogenic and non-embryogenic cultures share a common explant origin and are initiated and maintained under the same culture conditions, the presence of the 32 kDa chitinase-like protein in embryogenic suspension cultures only indicates that it is associated with somatic embryogenesis and that it can be possibly used as a marker for embryogenic potential. Van Hengel *et al*. (1998) and Wojtaszek *et al*. (1998) also found a chitinase constitutively expressed in *Lupinus* and carrot suspension culture in the absence of applied stress factors, respectively.

The embryogenic suspension cultures E_1, E_2 and E_3 consist of different cell types with different morphology and developmental potential (Tchorbadjieva and Odjakova 2001). To determine the morphological structures that secrete the chitinase-like protein, the extracellular proteins from subsequent developmental stages were subjected to SDS-PAGE and immunostained

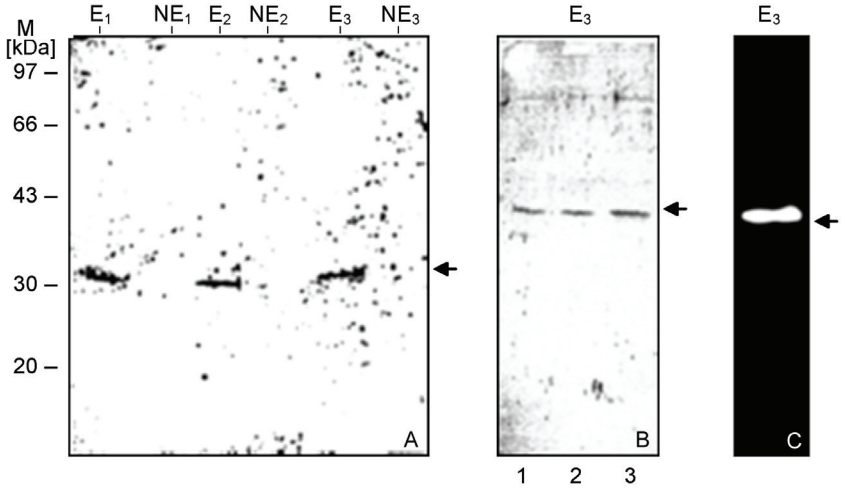

Fig. 1. Detection of a chitinase-like protein and its chitinase activity in culture media of *Dactylis glomerata* L. suspension cultures. *A* - Immunoblot with extracellular proteins from embryogenic (E_1, E_2, E_3) and non-embryogenic (NE_1, NE_2, NE_3) suspension cultures with anti-32 kDa chitinase serum. *B* - Immunoblot with extracellular proteins from: microclusters→PEMs (*lane 1*), PEMs→somatic embryos (*lane 2*) and mature somatic embryos (*lane 3*) of E_3 embryogenic suspension culture. C - Chitinase activity in extracellular proteins from PEMs of E_3 embryogenic suspension culture after 12 % SDS-PAGE with 0.01 % glycol chitin included in gel. The position of the 32 kDa chitinase-like protein and chitinase activity are indicated by the *arrows*. M - molecular mass standards in kDa.

with anti-chitinase EP3 antibody (Fig. 1*B*). A chitinase-like 32 kDa protein was found in the medium of all morphological structures and phases of somatic embryogenesis, which shows a constitutive expression of this protein during development. This is in agreement with Mo *et al*. (1996) who observed a correlation of chitinase secretion in a *Picea abies in vitro* culture with the ability of PEMs to form normal somatic embryos. Two chitinase isoforms were shown to accumulate in the medium of embryo cultures to a much higher level compared to their level in the medium of a non-embryogenic *Cichorium* cultivar (Helleboid *et al*. 2000).

We examined further the chitinolytic activity of the chitinase-like protein from the E_3 embryogenic cell line. One lytic zone (Fig. 1*C*) indicated the presence of one protein able to degrade the artificial chitinase substrate glycol chitin and it corresponded to the 32 kDa protein detected by the antibody in the E_3 embryogenic cell culture (Fig. 1*A*). 2-D gel electrophoresis and immunoblotting showed that the band of 32 kDa from 1-D separation of E_3 extracts (Fig. 1*B*, *lane 2*) resolved in a unique spot located in the acidic part of the electrophoregram (Fig. 2*A*). Silver staining of a duplicate gel showed that the corresponding 32 kDa protein (pI 3.6) is a relatively abundant one (Fig. 2*B*).

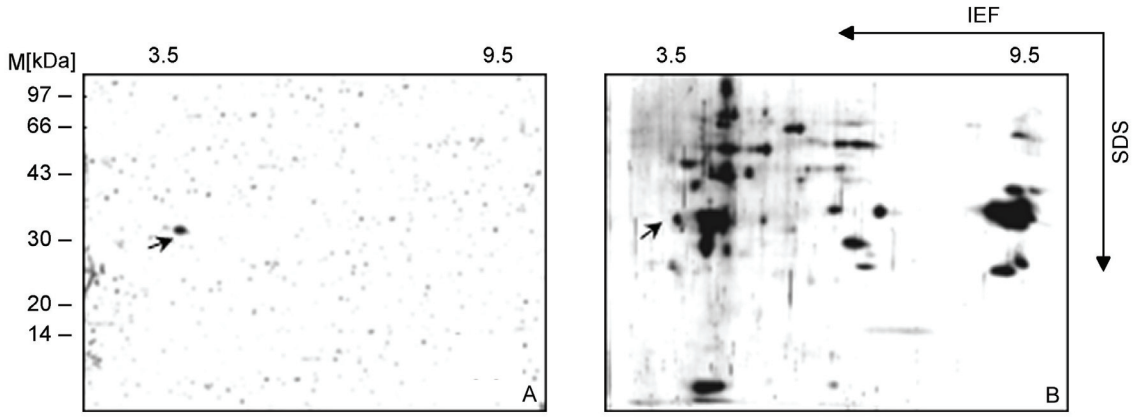

Fig. 2. Immunoreactivity of the extracellular proteins secreted by PEMs from E_3 embryogenic suspension culture with anti-32 kDa serum from carrot after 2-D gel electrophoresis. *A* - immunoblot; *B* - silver-stained duplicate gel of *A*. The 32 kDa acidic chitinase-like protein (pI 3.6) is shown with an *arrow*.

The chitinase biochemical function resides in the ability of some plant chitinases to degrade fungal cell walls (Collinge *et al*. 1993). Chitinase genes were also shown to be expressed in the absence of pathogens (De Jong *et al*. 1992, Van Hengel *et al*. 1998). Besides, the ability of chitinase-like proteins to cleave sugar chains of AGPs has been shown in embryogenic cell lines of pine (Domon *et al*. 2000) and carrot (Van Hengel *et al*. 2001). Thus, it can be stated that chitinases through participation in production of specific signal molecules may play a regulatory role in embryogenesis.

To summarize, we have found an acidic 32 kDa chitinase-like protein in embryogenic suspension cultures only during all stages of somatic embryogenesis of *Dactylis glomerata* L. suspension cultures. The full identity of molecular mass (32 kDa) and pI (3.6) between the chitinase-like protein in orchardgrass suspension cultures and the carrot endochitinase EP3 with ability to rescue somatic embryogenesis tempts us to speculate on a similar role in somatic embryogenesis. Future work will address the precise structure of this chitinase-like protein and its role in plant development.

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