

BRIEF COMMUNICATION

## Involvement of arabinogalactan proteins in the control of cell proliferation of *Cucurbita pepo* suspension cultures

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### Abstract

Arabinogalactan proteins (AGPs) secreted by zucchini squash (*Cucurbita pepo* L.) cell cultures into the medium are implicated in cell proliferation. Conditioned medium derived from cell suspensions of squash cultivar Dundoo could enhance multiplication rate of slow-growing cell line Cx3005. To examine the role of AGPs, a precipitation assay was performed using Yariv reagent which binds selectively to AGPs. This AGP precipitation as well as proteinase application arrested cell division. However, chitinase treatment successfully increased embryogenic callus mass. A growth promotion was also obtained by arabinogalactan addition to the culture medium. Immunoblotting analysis using the MAC 207 anti-AGP monoclonal antibody showed high AGP expression in Dundoo cell cultures.

*Additional key words:* chitinase, embryogenic cultures, extracellular proteins.

Cell suspension systems represent a good model for studying developmental regulation and understanding plant morphogenesis, signalling pathways and cell proliferation mechanisms. Compounds - such as peptides, oligosaccharides and glycoproteins - secreted into the liquid culture contribute to the conditioning of the medium (Matthys-Rochon 2005). The promoting effect of this medium for the establishment of new cell lines has been reported previously (Van Hengel *et al.* 1998, Ben Amar *et al.* 2007).

Cell wall proteins are currently being investigated for their function in molecular interactions and cell signalling (Majewska-Sawka and Nothnagel 2000, Jamet *et al.* 2006). Beside small peptides, conditioned medium obtained from plant cell cultures also contains some structurally complex macromolecules like arabinogalactan proteins (AGPs), identified as lectins, with often more than 90 % sugar content. AGPs are largely involved in a variety of plant growth aspects (Svetek *et al.* 1999) but their cellular localization remains unclear because of

extreme solubility (Showalter 1993). AGPs can be found as extracellular compounds secreted by cultured cells into the medium (Komalavilas *et al.* 1991, Van Hengel *et al.* 2001, Chassen and Blaschek 2002, Immerzeel *et al.* 2004, Ben Amar *et al.* 2007). These extensively glycosylated hydroxyproline-rich glycoproteins (HRGPs) are abundant extracellular matrix components and play major roles in developmental regulation including cell signalling (Pennell *et al.* 1989, Showalter 2001), cell elongation and cell wall extensibility (Willats and Knox 1996, Knox 2006), cell differentiation (Willats *et al.* 1999), reproductive development (Pennell and Roberts 1990, Sun *et al.* 2004, Qin and Zhao 2006), somatic embryogenesis (Van Hengel *et al.* 2001, Tang *et al.* 2006), stress tolerance (Lamport *et al.* 2006) and programmed cell death (Majewska-Sawka and Nothnagel 2000). However, few studies have assigned evidence for the involvement of AGPs on cell proliferation (Serpe and Nothnagel 1994, Kimberly and Nothnagel 1997, Kaparakis and Alderson 2003).

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*Abbreviations:* AG - arabinogalactan; AGPs - arabinogalactan proteins; CM - conditioned medium; 2,4-D - 2,4-dichlorophenoxyacetic acid; MES - 2-(*N*-morpholino)-ethanesulfonic acid; PVDF - polyvinylidene difluoride;  $\beta$ -GlucY -  $\beta$ -D-glucosyl phenylglycoside.

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The aim of the following experiments was to confirm assumption that AGPs secreted into the culture medium by zucchini squash cultured cells enhance cell division and embryogenic callus proliferation.

Cell suspensions of two *Cucurbita pepo* L. cultivars (Dundoo and Cx3005) were initiated with 0.5 g fresh mass of embryogenic callus grown on Murashige and Skoog (1962; MS) liquid medium containing 2 % (m/v) maltose, 0.46 % (m/v) glycerol, 0.05 % (m/v) MES, 5 mg dm<sup>-3</sup> 2,4-dichlorophenoxyacetic acid (2,4-D) and 3 mg dm<sup>-3</sup> kinetin. These suspensions were incubated in darkness at 24 °C on a gyratory shaker (90 rpm). Suspensions containing growing pre-embryogenic masses (PEMs) were maintained by weekly subculture and divided every 4 weeks.

Conditioned medium (CM) was obtained from well established cell cultures, harvested at the 8<sup>th</sup> day after the last subculture. Conditioned medium was centrifuged for 20 min at 10 000 g and the supernatant was filter sterilized (0.22 µm Millipore filter).

The proteinase K (*Carl Roth*, Karlsruhe, Germany) was used for protein inactivation in CM at the concentration of 30 U per 50 cm<sup>3</sup> CM for 6 h at 37 °C. The chitinase from *Streptomyces griseus* (*Sigma-Aldrich*, St.Louis, USA) was applied (1 U per 50 cm<sup>3</sup> fresh medium) at the time of culture initiation in order to examine possible involvement of polymer sugar residues in proliferation. Bioassays were also performed on suspensions supplemented with exogenous arabinogalactan from larch wood (*Sigma-Aldrich*) (1 mg per 50 cm<sup>3</sup> fresh medium). Specific precipitation of AGPs in the cell culture media was performed by addition of a dichlorophenoxyacetic acid synthetic phenyl glycoside (β-GlcY) (*Biosupplies PTY*, Sydney, Australia) known as Yariv reagent (Yariv *et al.* 1962) that interacts specifically with AGPs as described by Serpe and

Nothnagel (1994).

Cell suspension (0.5 cm<sup>3</sup>) was centrifuged, liquid supernatant was removed and 0.2 cm<sup>3</sup> of an *EasyPack* protease inhibitor mixture (*Roche Applied Science*, Mannheim, Germany) was added to each sample before grinding at 4 °C. After centrifugation at 7 500 g for 10 min, the supernatant of the cell extract was used for the experiments.

For the immuno-detection assay, CM was concentrated 100-fold using 3.5 kDa cut-off membrane (*Membra-Cell™ Dialysis*, Serva, Paris, France), then 0.02 cm<sup>3</sup> of each fraction were applied on a *Hybond™*-protein transfer membrane (*Amersham Biosciences*, Stockholm, Sweden) to perform a Dot-Blot using an *Easy-Titer® Elisa System* (Pierce, USA). The membrane was blocked in Tris-buffered saline (TBS) containing 1 % low-fat dried milk for 2 h, and then incubated overnight with the primary antibody. AGPs were detected by the specific monoclonal antibody MAC 207 (*CarboSource Services*, USA). Antibody binding was recognized by using a peroxidase conjugated anti-rat IgG antiserum (*Sigma*). The dot-blot was stained with a *Lumilight* Western blotting kit (*Roche Diagnostics*).

Initiation of cell suspensions from solid culture derived zucchini squash callus, often resulted in browning of the embryogenic material related to stress as well as in an arrest of cell growth (data not shown). However, using a medium that has been already conditioned by zucchini cells, proembryonic mass formation was very efficient (Fig. 1A). Embryogenic callus suspension of *C. pepo* cv. Dundoo initiated with CM showed vigorous cell proliferation and significant increase of fresh mass. Furthermore, using CM of Dundoo to establish a cell suspension of Cx3005 line showing a low growth rate, cell proliferation was induced resulting in the increase of callus fresh mass (Fig. 1A).

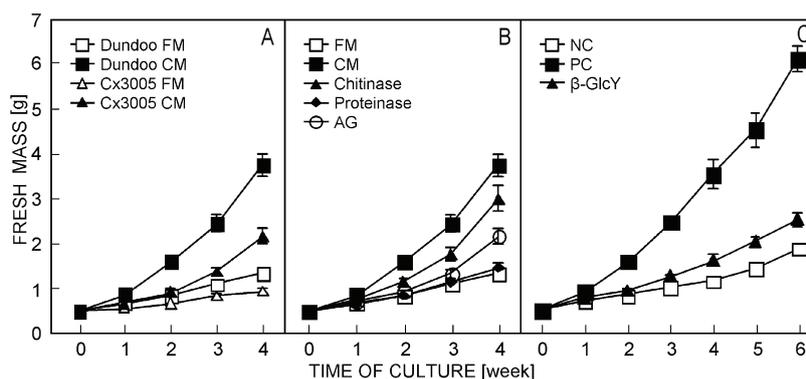


Fig. 1. *A* - Change in callus fresh mass during suspension initiation of zucchini squash (*C. pepo*) cv. Dundoo and cv. Cx3005. 0.5 g of embryogenic callus taken as starting inoculum was transferred into fresh liquid medium (FM) or into conditioned medium (CM) derived from Dundoo established suspension cultures. *B* - Time-course of growth in callus mass of squash cell suspensions (cv. Dundoo). Cell cultures were initiated either in the presence of proteinase K (30 U), chitinase (1 U) or arabinogalactan (AG; 1 mg) in the culture medium. Control suspensions grew in conditioned medium (CM) or in fresh medium (FM). The initial inoculum was 0.5 g of embryogenic material. *C* - Effect of β-GlcY reagent treatment on suspension of cv. Dundoo after 6 weeks of culture. Yariv reagent (50 µM) was applied at the beginning of the culture period to the conditioned medium (NC - negative control (FM), PC - positive control (CM)). Bars indicate SD values of 3 repetitions.

To investigate the nature of stimulating compounds present in CM, proteinase K was applied to degrade overall extracellular proteins soluble in the liquid medium as well as those attached to the surface of cell wall matrix. Using proteinase K, we observed a growth performance as low as in starting fresh medium (Fig. 1B). These results suggested that the substances secreted represent active proteins that were degraded by proteinase action. In contrast, addition of chitinase to the fresh medium enhance significantly cell growth. After 4 weeks, suspensions cultured in the presence of chitinase show more than 3 time higher cell proliferation compared to untreated cultures. This growth promoting effect of this enzyme might be due to the cleavage of oligosaccharide residues playing a role as signal molecules (Willats *et al.* 1999, Van Hengel *et al.* 2001, Kasprzewska 2003). Moreover, supplementing 1 mg of arabinogalactan from larch wood to the fresh medium at suspension initiation also showed a stimulating effect on embryogenic cell proliferation (Fig. 1B). Similarly, AG was shown to improve embryogenesis in wheat microspore culture (Letarte *et al.* 2006).

To determine whether the AGPs are implicated in the induction process of cell division, addition of Yariv reagent may disrupt AGP function and provide an insight into the role of AGPs.  $\beta$ -GlucY containing CM, used to initiate a new suspension culture, had a significantly reduced growth effect. Reduction in cell growth was proportional to the concentration of  $\beta$ -GlucY supplied into the medium (data not shown) and the highest inhibition of growth occurred at 50  $\mu$ M (Fig. 1C).

CM-derived AGPs were detected by immunoblotting analysis with the MAC 207 monoclonal antibody (Fig. 2). Positive signal were obtained with this antiserum and revealed clearly the presence of AGPs in the CM of *C. pepo*. Obviously concentration of AGPs in Dundoo suspension medium seems to be higher compared to Cx3005 cell lines.

There is no doubt, from the previous observations (Van Hengel *et al.* 1998, Matthys-Rochon 2005, Ben Amar *et al.* 2007) and the results reported in the present paper, about the effect of conditioned medium to promote embryogenic suspension growth. Conditioned medium derived from fast-growing embryogenic suspension of

cv. Dundoo has the ability to enhance growth not only of the same cultivar but also of cell line Cx3005 considered as recalcitrant. AGPs secreted by cv. Dundoo cells were found biologically active and also could affect proliferation of grape cell-aggregates (data not shown). This fact confirms our previous observations described in grapevine cell suspensions (Ben Amar *et al.* 2007).



Fig. 2. Dot blot of AGPs derived from cell extract (Ce) and conditioned medium (CM) of *Cucurbita pepo* cv. Dundoo and Cx3005 probed with MAC 207 monoclonal antibody. PC - arabic gum used as positive control, D-Ce - Dundoo cell extract, Cx3005-Ce - Cx3005 cell extract, D-CM - Dundoo conditioned medium, Cx3005-CM - Cx3005 conditioned medium, FM - fresh medium used as negative control.

Immunodetection of AGP from both CM and cell extract using an anti-AGP monoclonal antiserum was achieved. The high glycosylation level up to 90 % of total molecular mass inhibits the migration of AGPs in SDS-PAGE preventing their detection by Western blotting (Mau *et al.* 1995). Direct application by dot blotting on PVDF membrane, however, allowed the immunodetection. Presence of signals after blotting with the MAC 207 antibody indicated the occurrence of AGPs in the cells as well as in the culture medium. The *L*-arabinose/*D*-glucuronic acid epitope recognized by the MAC 207 has been also found on an AGP soluble proteoglycan secreted into the medium by carrot suspension culture cells (Pennell *et al.* 1989). AGPs appear to be present in cell extracts of both squash cultivars. However, culture medium of Dundoo contained more soluble AGPs compared to that of Cx3005, which could be responsible for a better cell proliferation rate of Dundoo embryogenic suspensions.

Further investigations should be carried out for the complete understanding of the molecular mechanism by which AGP organisation at the cell surface form signal to control cell division process.

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