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REGULAR ARTICLE

ARABINOGALACTAN PROTEINS IN EMBRYOGENIC AND NON-EMBRYOGENIC MAIZE CALLI.

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ABSTRACT

Maize is one of the world's three most widely cultivated crops (along with wheat and rice) and it is the most economically important cereal crop on a worldwide basis. Tissue culture and embryogenesis induction from somatic cells are the main techniques necessary for practical application of advanced biotechnological tools for targeted improvement of the plant. Technical developments within this field of biotechnology have allowed greater reproducibility of experimental results and numerous investigations have demonstrated the utility of these techniques in the study of many biological topics, especially in genetics, plant physiology and development. In our experiments, callus induction and plant regeneration were tested using immature embryos of maize (*Zea mays* L.). Yield of embryogenic callus was highest when immature embryos were used as a primary explant and reached level of 10%. Protein extracts from embryogenic and non-embryogenic calli were used for western dot blot with specific antibodies against three epitopes of arabinogalactan proteins (antibodies JIM 8, JIM 13 and LM 2). These epitopes are considered as markers for embryogenic cells in various species. We have detected presence of arabinogalactan protein epitopes in extract from embryogenic callus, while extract from nonembryogenic callus showed only weak or no signal after western dot blot.

Keywords: Embryogenic callus, Non-embryogenic callus, Maize (*Zea mays* L.), Arabinogalactan proteins

INTRODUCTION

Maize (*Zea mays*, L.) is worldwide the third most planted cereal crop after wheat and rice. The production of this crop is on the decrease due to increased population, limited land, environmental and biotic stresses. Over the years, conventional breeding has been used as a tool to overcome these constraints (**Binott et al., 2008**).

Somatic embryogenesis is developmental process by which somatic cells undergo restructuring to generate embryogenic cells. These cells then go through a series of morphological and biochemical changes that result in the formation of somatic or non-zygotic embryo capable of regenerating plants. Somatic embryogenesis represents an unique developmental pathway that includes a number of characteristic events: dedifferentiation of cells, activation of cell division and reprogramming of their physiology, metabolism and gene expression patterns (**Yang et al. 2011**). Somatic embryogenesis forms the basis of cellular totipotency that is unique to higher plants (**Zimmerman, 1993; Schmidt et al., 1997; Komamine et al., 2005**).

Early somatic embryogenesis involves differentiated somatic cells acquiring embryogenic competence and proliferating as embryogenic cells. Initiation of the embryogenic pathway is restricted to certain responsive cells that have the potential to activate genes involved in generating embryogenic cells. Once these genes are activated, an embryogenic gene expression program replaces the established gene expression pattern in the explant tissue **(Quiroz-Figueroa et al., 2006).** Determining specific physical and chemical factors that switch on the embryogenic pathway of development is a key step in embryogenic induction. It has been proposed that plant growth regulators (PGRs) and stresses play a central role in mediating the signal transduction cascade leading to the reprogramming of gene expression, followed by a series of cell division that induce either unorganized callus growth or polarized growth leading to somatic embryogenesis **(de Jong et al., 1993).** Activation of auxin responses may be a key event in cellular adaptation and genetic, metabolic and physiological reprogramming, leading to the embryogenic competence of somatic plant cells. Outside of auxin as a main inducer **(Fehér et al., 2003)**, there have been reports for somatic embryogenesis in response to either other growth regulators such as cytokinin **(Sagare et al., 2000)** or abscisic acid (ABA) being present or absent **(Senger et al., 2001).**

Since proteins directly influence cellular biochemistry and provide a more accurate analysis of cellular changes during growth and development **(Chen and Harmon, 2006),**

identification of proteins associated with somatic embryo development may provide mechanistic insight onto somatic embryogenesis.

Both cytoskeleton and structural components of cell wall play regulatory role during initial steps of embryo development in vitro. Embryogenesis is controlled by cell wall molecules, mainly pectines and arabinogalactan proteins **(Šamaj et al. 2006).** A network of fibrillar material forming outer continuous layer covers groups of early proembryogenic cells. This layer is called extracellular matrix surface network and has been found in maize embryogenic callus **(Šamaj et al. 2006)** as well as in maize microspore derived embryogenic structures **(Obert et al. 2005).** Additionally this extracellular matrix surface network was described in many dicotyledonous, monocotyledonous and gymnosperm plant species **(Obert et al. 2010)**, thus might be considered as a general, and conserved structural marker of competent merristematic cells having morphogenetic potential. Some components of extracellular matrix network has been identified as arabinogalactan proteins and pectines **(Šamaj et al. 1999, Chapmann et al. 2000).** Further studies confirmed several epitopes of arabinogalactan proteins as a structural marker of embryogenesis **(Šamaj et al. 2006).**

MATERIAL AND METHODS

Source plants and preparation

Maize callus induction system was developed using immature embryos. Two maize inbred lines (A18 and A19) were used in our experiments. Plants were grown in experimental field of the Institute of Plant Genetics and Biotechnology in Nitra. Ears were harvested 16 and 20 days after pollination. Size of immature embryos was 1- 4 mm. The ears were surface sterilized for 5 min in 70% ethanol and then for 20 min in 2% sodium hypochlorite. This was followed by triple rinsing in sterile distilled water. Immature embryos were aseptically isolated by cutting the tips of the kernels with a scalpel without touching the embryo **(Jakubeková et al., 2012).**

Media components

Media for callus cultures was callus initiation medium N6 (**Chu et al., 1975**) plus 2 % sucrose, 25 mmol.L^{-1} proline, 1 mg.L^{-1} 2,4-D, 100 mg.L^{-1} casein hydrolysate, N6 vitamins, 10 mg.L^{-1} silver nitrate, 3g gelrite. The media was adjusted to pH 5,8 and autoclaved. Cultures were incubated at 28 ºC in the dark. Cultures were transferred every 14 days to fresh medium but without silver nitrate.

Type of induced callus (embryogenic, non-embryogenic and organogenic) was determined by examining under the binocular stereomicroscope Leica ZOOM 2000 (Germany) and later on by ability to form appropriate structures. Embryogenic callus was transferred into embryo maturation medium containing N6 medium supplemented with N6 vitamins, 6% sucrose, 1 $mg.L^{-1}$ NAA and 3g gelrite for embryo maturation. Cultures were incubated in the dark at temperature of 28 ºC **(Jakubeková et al., 2012).** For regeneration, friable callus with somatic embryos was transferred to fresh medium containing MS medium (**Murashige and Skoog, 1962**) supplemented with 2% sucrose, 2 $mg.L^{-1}$ myo-inozitol, 3g gelrite for plant regeneration and moved from 24-h darkness in the light. Well-rooted plantlets from culture flasks were rinsed with water to remove the media then transferred into pots containing peat moss.

Protein extraction

Embryogenic and non-embryogenic callus were used in proteomic analysis. Protein extraction was performed as described **Hurkman and Tanaka (1986)** with some modifications. Plant material was grounded in liquid nitrogen using mortar and pestle. Then 5 ml extraction buffer (0,1 M Tris-HCL pH 8,8; 10mM EDTA; 0,9 M sucrose), 20 µl 2-mercaptoethanol and 5 ml phenol was added to each mortar. The mixture was transferred to a 10 ml tube and centrifuged at 4000 x g for 10 min at 4 °C . Proteins from the phenol phase were precipitated by adding five volumes of pre-chilled 0,1 M ammonium acetate in 100% methanol and incubated overnight at -20 ºC. The precipitate was collected by centrifugation for 20 min., 4000 x g at 4 ºC. Finally, the pellet was washed 2 times with 0,1 M ammonium acetate in methanol, 2 times with ice-cold 80 % acetone and finally 1 time with cold 70 % ethanol. After a brief air-drying, the protein pellet was re-suspended in rehydration buffer 1h (8 M urea, 2 M thiourea, 2 % CHAPS).

Western dot blot

Protein extracts from embryogenic and nonembryogenic calli were transferred to a PVDF (polyvinylidene difluoride) membrane. Membranes were blocked for 2 hours in a Tris/powdered milk solution. The optimal antibody concentrations were found by serial dilution so that resulting dot intensities were within visible range of detection. Membranes were incubated overnight at room temperature with appropriate dilution of the antibodies JIM 8, JIM 13 a LM2. After overnight hybridization with primary antibody, membranes were

incubated with alkaline phosphatase conjugated secondary antibody. Signal was developed with the alkaline phosphatase substrate.

RESULTS AND DISCUSSION

Callus initiation from immature embryos was observed after the seventh day of culture on callus initiation medium from the scutellum. This was due to the presence of meristematic cells in the scutellum **(Jakubeková et al., 2012).** Several experimental observations differentiated fate of plant cells, dependent on positional information and developmental signals, can be easily altered under *in vitro* conditions. In vitro tissue culture conditions expose the explants to significant stresses, as they are removed from their original tissue environment and placed on synthetic media containing non-physiological concentrations of growth regulators, salts and organic components **(Fehér et al., 2003).**

The percentage of the primary callus formed to 95% from immature embryos, while embryogenic callus was formed in frequency from 2 to 5%. Embryogenic callus was formed after the first subculture on callus maintenance medium. Somatic embryos were initiated on the surface of the embryogenic callus. Three types of callus were formed: embryogenic, non-embryogenic and organogenic callus. Embryogenic callus was compact and light yellow formed. Non-embryogenic callus was soft, watery and yellow in color, which ceased to grow and turned brown in subsequent subculture. From organogenic callus mainly roots were formed **(Jakubeková et al., 2012).**

Hormones regulate the developmental switches. Auxins and cytokinins are the main growth regulators in plants involved in the regulation of cell division and differentiation **(Fehér et al., 2003).** 2,4-dichlorophenoxyacetic acids (2,4-D) is exogenously applied auxin on the induction of somatic embryogenesis **(Carvalho et al. 1997; Bohorova et al., 1995).** The optimum 2,4-D concentration for the initiation of embryogenic callus was 1 mg. L^{-1}. High concentration of 2,4-D applied to induction media reduced the percentage of embryogenic callus formed suggesting that 2,4-D had inhibitory effect at higher concentration **(Jakubeková et al., 2012).**

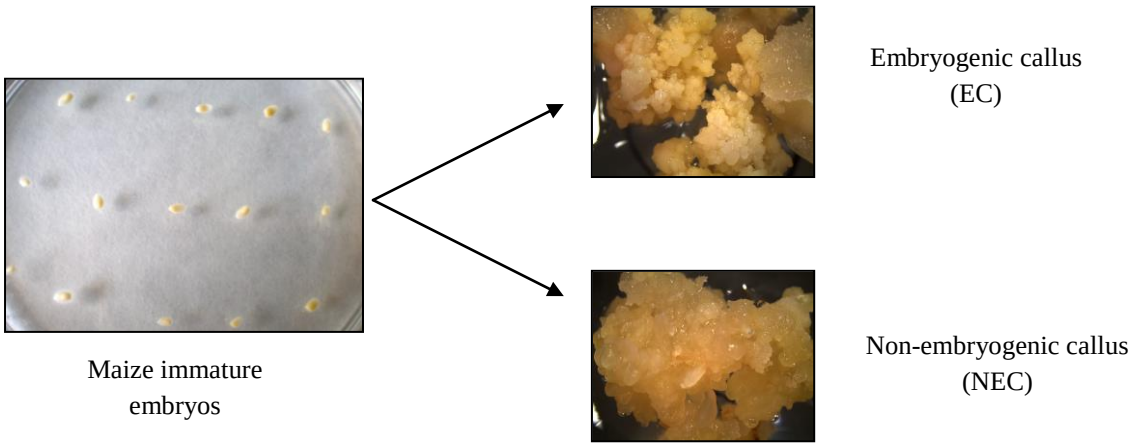

Fig. 1 Maize somatic embryogenesis. Immature embryos were used as the initial explants. SE was induced in medium Nitsch containing auxin (2,4-D). Immature embryos produced both embryogenic callus (EC) and non-embryogenic callus (NEC).

It is well known that arabinogalactan proteins are developmentally regulated in reproductive organs and during seed and vegetative development **(Šamaj et al.2006).** It was revealed that several epitopes of arabinogalactan proteins are developmentally regulated during embryogenesis in various species **(Obert et al 2010).** These epitopes can serve as specific molecular markers for embryogenic cells in diverse species. However in maize some arabinogalactan proteins epitopes are not so specific and they are present in both embryogenic and non-embryogenic cells as it is case of Gal 4 and JIM 15. Nevertheless, they might show distinct preferences for certain cell types as it is the case for Gal 4 which labels embryogenic cells weakly in spot like manner, while differentiated cells show stronger labeling at the plasma membrane and at some intracellular compartments **(Šamaj et al 2006).** Results of arabinogalactan proteins in protein extract from embryogenic and non-embryogenic maize calli detection are presented in figure 2. In protein extract from embryogenic calli was clearly detected positive signal with antibodies JIM 13 and LM 2, while non-embryogenic calli showed just weak signal with antibodies JIM 8 and LM 2. These findings are in agreement with specificity of arabinogalactan proteins epitopes JIM 13 and LM 2 to embryogenic cells. Thus antibodies JIM 8 and LM 2 can be used as a marker for embryogenic cells in maize.

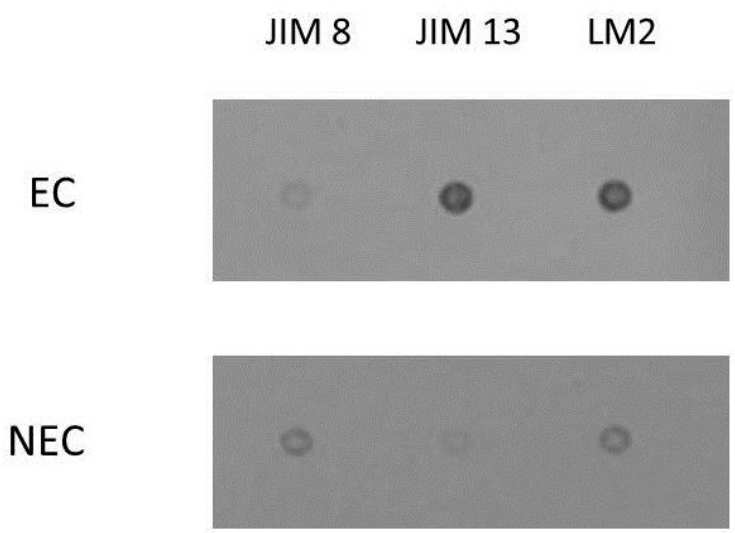

Fig. 2 Western dot blot of protein extracts from embryogenic (EC) and non-embryogenic (NEC) maize calli with arabinogalactan ptoreins. In embryogenic callus was clearly detected positive signal with antibodies JIM 13 and LM 2, while non-embryogenic callus showed just weak signal with antibodies JIM 8 and LM 2.

CONCLUSION

Somatic embryogenesis is an important in vitro technique for the multiplication of maize plants. This method consists of developing embryoids from haploid cells or somatic diploids, without gamete fusion, allowing an accelerated micropropagation of superior clones and the maintenance of interspecific hybrids (**Tonietto et al. 2012**).Somatic embryogenesis is an important step in any successful plant transformation scheme, because each transformed cell of the callus has the potential to produce a whole plant.

The objective of this work was to analyze several epitopes of arabinogalactan proteins in protein extract of embryogenic and non-embryogenic maize calli. We have confirmed that arabinogalactan proteins are connected with embryogenic development. Antibodies JIM 13 and LM 2 can be used as molecular marker for embryogenic cells in maize.

Further we will concentrate on proteome of embryogenic and non-embryogenic maize calli analysis and comparison. The differential gene expression in somatic cells is involved in developmental reprogramming of these cells, and confers the capacity to manifest the embryogenic potential (**Zeng et al. 2006**). Through this approach, it is possible to obtain better adapted cultivars with a high multiplication rate when compared to other propagation

methods. It is also possible to transfer genes and therefore, this method is frequently used in genetic improvement studies of clonal propagation (**Tonietto et al. 2012**).

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