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FLAX (*LINUM USITATISIMUM* L.) – A PLANT SYSTEM FOR STUDY OF EMBRYOGENESIS

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Embryogenesis is a critical stage of the sporophytic life cycle, during which the basic body plan of the plant is established. Although zygotic embryogenesis is induced by fusion of the sperm and egg nuclei, plant cells can initiate embryo development without fertilization. For example, cultured somatic and male gametic cells can be induced to undergo somatic and microspore embryogenesis, respectively. Embryogenesis in vitro represents a powerful tool to manipulate plant development. After characterizing in situ embryo development in flax, we followed the cytological, morphological and some biochemical features of zygotic embryo development in embryo cultures. We also induced direct and indirect somatic and gametic embryo formation in flax. There is a strong indication that somatic and gametic embryogenesis is a stress response and that it is a way the plant cell realizes its survival strategy under completely changed and unusual conditions.

Key words: *Linum usitatissimum* L., zygotic embryogenesis, somatic embryogenesis, gametic embryogenesis, embryo-like structure.

INTRODUCTION

Embryogenesis in higher plants begins with a double fertilization event in which two sperm nuclei fuse with the egg cell and central egg nuclei to initiate embryo and endosperm development. The zygote then undergoes a series of cell divisions and differentiation events to produce the mature embryo (West and Harada, 1993; Yadegari and Goldberg, 1997; Laux and Jürgens, 1997; Berleth, 1998).

Embryogenesis can also arise from somatic or gametic (microspore) cells (Preťová and Williams, 1986; de Vries et al., 1988; Cordewener et al., 1994) either naturally, as has been observed in *Kalanchoë* where somatic embryos form spontaneously on leaf edges, or in vitro after experimental induction. Acquisition of embryogenic competence in somatic and gametic embryogenesis involves an induction phase for which there is no counterpart in zygotic embryogenesis (Preťová, unpublished data). The term "embryogenic cell" would be limited to cells that have achieved the transition from a somatic cell to a stage in which no further external stimuli are required to produce a somatic (or gametic) embryo (Komamine et al., 1990). These two different types of embryogenesis have been analyzed in different model plant species (e.g., *Arabidopsis, Daucus carrota*). This paper introduces a plant species in which all described modes of embryo formation can be studied, and stresses some special features we observed in different flax embryogenic systems.

Flax (*Linum usitatissimum* L.) attracts interest as a diversification crop for oil and fiber production in European and world agriculture (Preťová et al., 2001; Preťová and Obert, 2001). Zygotic embryo development in flax (Preťová, 1977; 1978; Preťová and Vojteková, 1985; Vizárová et al., 1987; Preťová et al., 2001) and flax embryo development in vitro

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(Pret'ová, 1974; Pret'ová, 1986; Pret'ová and Williams, 1986; Dedičová et al., 2000; Pret'ová et al., 2000) have been studied intensively in recent decades.

FLAX EMBRYOGENESIS

Embryogenesis in vitro is represented by embryo cultures of young zygotic embryos, somatic embryogenesis and gametic (microspore) embryogenesis. By subtle manipulation of growth hormones such as auxins and cytokinins supplied to the culture medium, and by adjusting culture conditions, embryo development can be induced and/or promoted and complete plantlets can be regenerated. Embryo cultures are the basis for all three mentioned areas of in vitro embryogenesis. After the induction phase in systems of somatic and gametic embryogenesis, we have to deal with embryo culture again.

In the first 24 h in culture, the young (from globular stage onward) zygotic flax embryo enlarged 25–30% as a result of balancing water deficit and osmotic conditions. Afterwards, limited proportional growth (reduced cell division) was observed, followed by a short lag-phase (2–3 days). Then intensive cell division and growth occurred during the next 5–7 days in culture (Pret'ová, 1978; 1990). Formation of cotyledons was a crucial stage of embryo development in culture when globular zygotic embryos were cultured. In embryo cultures, 20–25% of globular embryos, 60–65% of heart-shaped embryos and 85–90% of older stages were capable of completing the embryo developmental program in vitro, resulting in a cotyledonary stage embryo with a conspicuously prolonged hypocotyl region, ready to germinate. Adding glutamine significantly promoted flax embryo development in vitro, as young embryos lack nitrite- and nitrate-reductase enzymes (Pret'ová, 1990).

DIRECT SOMATIC EMBRYOGENESIS

Direct somatic embryogenesis in flax (*Linum usitatissimum* L.) induced from late heart or young torpedo zygotic embryos was first published in 1986 (Pret'ová and Williams, 1986) and was promoted by BAP and yeast extract. BAP seemed to be responsible for preserving the mitotic stimulus in the hypocotyl cells of the original zygotic embryo. Yeast extract inhibited further development of the shoot-root axis of the original zygotic embryo and preserved the internal pro-embryogenic determination of the initiating cells. Somatic embryos formed in a ring beyond the cotyledons of the original zygotic embryo (Pret'ová and Williams, 1986; Pret'ová, 1990) from the sub-epidermal cell layer (unpublished data). Later, somatic embryo formation was reported in *Linum alpinum* L. from protoplasts (Ling and Binding, 1987; 1992) and from flax seedlings grown in vitro (Gomes et al., 1996; Cunha and Fernandes-Ferreira, 1999).

We obtained direct somatic embryo formation also from 2 mm hypocotyl segments of 6-day-old flax seedlings cultivated on liquid MS medium with the addition of 2,4-D (2 mg l^{-1}). These structures were formed on the cut ends of segments within 14 days. Up to 18 embryo-like structures (ELS) per segment formed. Small heart-shaped embryo-like structures were freed to the culture medium and developed further. Approximately a third of those ELS reached the cotyledonary stage with well-formed shoot apices and were able to germinate. The rest of them did not have well-defined shoot apices; instead, secondary embryogenic structures were formed, or sometimes they possessed malformed (coalesced) cotyledons. All embryo-like structures were light green (unpublished data).

INDIRECT SOMATIC EMBRYO FORMATION

Somatic embryos were induced indirectly from older stages of zygotic flax embryos (20–28 days old) through a callus phase. For callus induction, the growth hormone 2,4-D in 2 mg l^{-1} and 5 mg l^{-1} concentrations was used. Callus was subcultured 3 or 4 times in short (2-week) subcultures to promote cell division and through this to reach dedifferentiation of cells. Finally, after this treatment the cells in the callus were able to express their totipotency and form ELS after subculturing on auxin-free medium. After such treatment, mass formation of ELS could be observed. During the process of indirect formation of flax somatic embryos, elements of extracellular matrix were observable by SEM. Elements of extracellular matrix were found on granular calli prior to ELS formation (Dedičová et al., 2000). The bipolar ELS often had abnormal morphology, and only rarely formed normal mature plants. We assume that polar auxin transport was severely disturbed in our system of indirect ELS production in flax. ELS were formed on the callus surface as well as inside the callus mass (Šamaj et al., 1997; Dedičová et al., 2000).

Our experiments showed that the cells of young zygotic embryos (heart- and torpedo-shaped) were already proembryogenically determined at the moment of culturing and needed only cytokinin to be able to form somatic embryos. The hypocotyl subepidermal cells of cultured zygotic embryos behaved somewhat like PEMs.

The cells of older zygotic embryos, highly differentiated and filled with reserve materials, were able to express their totipotency after treatment with 2,4-D and after dedifferentiation. Flax somatic embryos formed via either direct or indirect somatic embryogenesis showed several characteristics similar to those shown when very young (globular and heart-shaped) zygotic flax embryos developed in culture. The size of the differentiated somatic embryos was comparable to the enlarged and elongated cultured zygotic embryos. Considerable elongation of the hypocotyl region due to additional mitotic activity in the hypocotyls and the base of the radicle could be observed in both the somatic embryos and in zygotic embryos developing in culture. In both systems a higher degree of maturation of vascular elements was observed. The described phenomena can be considered a specific adaptation or response to in vitro conditions. The next similarity between the two systems can be seen in the formation of cotyledons, a crucial moment in both systems. Coalesced cotyledons formed very often. Somatic embryos derived from hypocotyl segments of flax seedlings did not have properly differentiated shoot apices and did not germinate well. They formed secondary embryogenic structures.

GAMETIC (MICROSPORE) EMBRYOGENESIS

Gametic (microspore) embryogenesis in flax was induced on N6 culture medium, preferentially via callus formation. The first microspore-derived calli appeared on anthers after 3 weeks in culture. Callogenesis was not synchronous, and callus formation varied in intensity depending on the cultivars and culture conditions used. Generally 1–2 microspore-derived calli were obtained from one responding anther. Granular calli varied in size and color (white-yellow, yellow or yellow-green, and light green). Shoots and/or embryo-like structures appeared on calli within the next week and could be transferred to a regeneration medium (Preťová and Obert, 2001). Direct formation of embryos from microspores cultured inside the anthers was observed only very sporadically (unpublished data). Only microspores in early and late uninucleate stages were responsive to induction treatments (8˚C for 7 days, 1mg l^{-1}NAA and 1mg l^{-1} BAP). The morphology of embryos originated from cultured microspores was similar to that of embryos derived from somatic tissues.

We believe that embryogenesis induced in vitro, both somatic and gametic, is a stress response. It can be understood as a survival strategy for cells of particular plant species under extremely altered and unfavorable conditions (in vitro culture). In very negative conditions the cells switch to the embryogenic developmental pathway, and the internal environment of the cell (or cells) changes to conditions similar to those in the zygote. By applying growth hormones and other stimuli we can make the conditions better for realization of this special developmental program.

Only the formation of an embryo structure can guarantee the preservation and continued survival of a plant species. Evolutionarily this ability to reproduce from a somatic or gametic cell can be considered an important survival strategy and an adaptation to predation, since plants are stationary and do not always have well-developed defense mechanisms. Furthermore, as a survival strategy the ability to mount an embryogenic response depends on the specific metabolic state of a cell or cells with a defined genetic potential to activate the proper set of genes involved in generation of embryogenic cells.

All three modes of embryo development in vitro considered in this contribution – culture of young zygotic embryos, somatic and gametic embryogenesis – enable study of the correlations and signalling pathways that control the process of embryo formation. Embryogenesis in vitro generally fulfills the basic role of embryogenesis in higher plants by differentiating meristems, forming the shoot and root axis, and differentiating primary tissues and tissues specialized for accumulation of reserve materials. The process ends with a structure prepared to develop into a complete plant.

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