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SECRETED MOLECULES AND THEIR ROLE IN EMBRYO FORMATION IN PLANTS: A MINI-REVIEW

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This short review emphasizes the importance of secreted molecules (peptides, proteins, arabinogalactan proteins, PR proteins, oligosaccharides) produced by cells and multicellular structures in culture media. Several of these molecules have also been identified in planta within the micro-environment in which the embryo and endosperm develop. Questions are raised about the parallel between in vitro systems (somatic and androgenetic) and in planta zygotic development. A view of exchanges between embryonic and nonembryonic multicellular structures in vitro is presented, and several facts about embryo and endosperm molecular interactions in planta are reported. Analysis of in vitro mechanisms may help in understanding what happens during zygotic embryogenesis.

Key words: Secreted stimulating factors, signaling molecules, tissue culture, zygotic, somatic, androgenetic embryogenesis, endosperm-embryo interrelations.

INTRODUCTION

Embryo development in plants starts after double fertilization and leads to the formation of an embryo and a second structure called the endosperm. They are both situated within the growing seed. Although numerous studies have been done, it is still unknown how an embryo is formed (Goldberg et al., 1994; Matthys-Rochon et al., 1997; Matthys-Rochon, 2002). Complex mechanisms operating during embryogenesis enable the development of a new plant. One obstacle in studying this development is the positioning of the embryo and endosperm within the maternal tissues. They are difficult to access for experimental manipulation, especially in very early stages of development.

One possible way to overcome this difficulty is to study what happens during in vitro culture, such as somatic and pollen embryogenesis. Somatic embryogenesis is the process by which asexual (somatic) cells develop into plants under in vitro conditions (Zimmerman, 1993; Mordhorst et al., 1997). Pollen or microspore embryogenesis, also referred to as androgenesis, is a method of developing haploid embryo after stress treatment (Touraev et al., 1997; Goralski et al., 1999). After cell multiplication and differentiation, these two systems generate embryos and subsequently plantlets without fusion of gametes as in zygotic development. In these in vitro cultures, the environment of the cells mimics the conditions that exist in ovulo.

Besides hormones known to stimulate embryo formation (Zimmerman, 1993; Matthys-Rochon et al., 1998), other classes of molecules have been identified as embryo-stimulating factors, especially those secreted into the culture medium. For many years, cell and tissue culture researchers have recognized the benefits of conditioned medium, in which cells have been grown previously, for the establishment of new cell lines or embryos (Halperin, 1966; Hari, 1980).

This review will focus on several groups of molecules that have a promoting effect on in vitro embryo development and which have been identified sometimes during zygotic seed formation. Then basic zygotic development will be compared with in vitro development in terms of the interrelations between embryo and endosperm, and between their in vitro counterparts.

OLIGOSACCHARIDES

The cell walls in plants are complex structures of multiple carbohydrates and proteins. In addition to its role as a structural framework, the cell wall serves as

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an extracellular matrix that contains signaling information for plant growth and development (Chasan, 1992; Mohnen and Hahn, 1993; Fry et al., 1993; Strauss, 1998; Sheen et al., 1999; Malinowski and Filipecki, 2002). Oligosaccharides derived from the plant cell wall have been identified in conditioned medium in different cell suspension cultures, including spinach (Fry, 1986), maize (Paire et al., 2003; Goralski et al., 2002), tobacco (Sims et al., 1996) and rice (Inui et al., 1996).

It is generally thought that the production of oligosaccharides in culture medium is the result of carbohydrate or glycoprotein splitting by cell wall enzymes. The resulting oligosaccharides might contribute to the nutrition of the developing structures, but also to the control of the embryogenic process. Thus, endogenous Nod-factor-like signal molecules have been shown to promote early somatic embryo development in Norway spruce (Dyachock et al., 2002). Wiweger et al. (2003) demonstrated in this gymnosperm that lipophilic low molecular weight molecules with Glc Nac residues are secreted by embryogenic cultures. The data indicate that lipo-chitinooligosaccharides (LCOs) homologous with rhizobial Nod factors are present in plants and stimulate proliferation of proembryogenic masses and somatic embryo formation (Dyachock et al., 2002).

PEPTIDES

Signaling peptides have been identified and well studied in animals and more recently in plants (Ryan et al., 2002). In plant cultures, only a few molecules are known which act on cell division and/or embryo development. Phytosulfokines (PSKs) are disulfated 4-5-amino-acid peptides first isolated from *Asparagus* cell suspension culture medium (Matsubayashi and Sakagami, 1996). In carrot somatic embryogenesis, Hanai et al. (2000) demonstrated the presence of PSK in conditioned medium of embryonic cell culture, and the stimulating action of this sulfated peptide on embryo formation. The authors showed that PSK is not capable of inducing embryogenic competence but that its addition to an embryo-inducing culture increased the number of cells and accelerated somatic embryo formation. PSK also stimulates somatic embryogenesis in *Cryptomeria japonica* (Igasaki et al., 2003). The putative receptor proteins for this autocrine-type growth factor were identified by photoaffinity labelling of plasma membrane fractions derived from rice suspension cells (Matsubayashi and Sakagami, 2000), and more recently a PSK receptor has been purified from membrane fractions of carrot cells (Matsubayashi et al., 2002). Overexpression of this receptor-like kinase in carrot cells enhances callus growth in response to PSK and substantially increases the number of PSK-binding sites, indicating that PSK and this receptor-like kinase act as a ligand-receptor pair. Now that the in vitro function of PSK and the molecular basis of ligand-receptor interaction in PSK signaling have been established, the next phase of research is characterization of the in vivo role of PSK and its downstream signaling pathway in plants (Matsubayashi et al., 2002). This study will provide the means to elucidate the mode of action of these PSKs in embryogenesis in vitro.

Important studies have been done on polypeptide hormones in plants (for review: Ryan et al., 2002). In particular, the amino acid sequence of the polypeptide RALF (Rapid Alkalinization Factor) has been determined. A search of databases revealed the existence of RALF homologs in more than 15 plant species. This polypeptide seems to have a role in development, especially in the germination of seeds. Although the function of these novel peptides remains unknown, experiments suggest their role may be developmental. We may thus suppose that this molecule acts on embryo development (Pearce et al., 2001; Haruta and Constabel 2003).

ARABINOGALACTAN PROTEINS

Media conditioned by plant cell cultures contain the small molecules mentioned above, but also larger ones like arabinogalactan proteins (AGPs) and pathogenesis-related proteins (PR proteins). They have been supposed to have a role in embryo formation.

The AGPs are a family of proteoglycans with very high carbohydrate content (90–98%) and consisting of high levels of arabinosyl and galactosyl residues and branched structures. In addition, their protein core is rich in hydroxyproline, serine, alanine and glycine (van Engelen and de Vries 1993; Showalter, 2001; Majewska-Sawka and Nothnagel, 2000). AGPs are detected with specific antibodies (Knox, 1997; Knox et al., 1991; McCabe et al., 1997; Pennel et al., 1989; Smallwood et al., 1995) or with β-Yariv reagent which binds to AGP and can be used as an inhibitor (Yariv et al., 1962). Briefly, in somatic embryogenesis, AGPs have been identified in cell culture medium of carrot (Kreuger and van Holst, 1993; McCabe et al., 1997), *Chichorium* (Chapman et al., 2000), rose (Svetek et al., 1999) and haploid cell cultures of barley (Paire et al., 2003) and maize (Borderies et al., 2004) microspores. Variation in the quantity of AGPs, their increase and subsequent decrease during the course of culture, has suggested that they are developmentally regulated, and it has been demonstrated that embryo development is inhibited if their action is blocked by Yariv reagent (van Hengel et al., 2002; Borderies et al., 2004). It is important to note that AGPs have also been detected in zygotic development of carrot and maize (van Hengel et al., 2002; Borderies et al., 2004), and they are secreted into cultures of endosperm cells (Gleeson et al., 1989).

PATHOGENESIS-RELATED (PR) PROTEINS

The enzymes (PR proteins; van Loon, 1990) that have been detected in the cell culture media are mainly glucanases, chitinases and thaumatins. In embryonic suspension cells of barley, Kragh et al. (1991) isolated three chitinases and one β-1,3-glucanase, some of them having similarities to those present in barley grain. β-1,3-glucanases have also been identified in *Chichorium* somatic embryogenesis (Helleboid et al., 1998, 2000), and to our knowledge the only example of the presence of β-1,3-glucanases in haploid development has been shown in maize (Borderies et al., 2004). It is known that the cell wall around embryonic cells contains callose. The callose deposition disappears as embryos grow. For this reason, it has been hypothesized that the culture medium accumulates β-1,3-glucanases, which may be responsible for degradation of the callose in the cell wall of embryogenic cells. The corresponding genes of these enzymes have been cloned, and their expression supports the hypothesis of a positive role of this enzyme during somatic embryogenesis in *Chichorium* (Helleboid et al., 2000).

Another interesting example is the determination and expression of *Chia4-Pa* chitinase genes during both somatic and zygotic development in Norway spruce (*Picea abies*). The presence and similarities of chitinases in angiosperms and gymnosperms have been reported, and a correlation between the increase of activity of *Chia4-Pa* genes and the induction of somatic embryos has been demonstrated (Wiweger et al., 2003). Recently the presence of chitinases has been detected in conditioned media of maize microspore culture, but at the moment nothing is precisely known about their role (Borderies et al., 2004).

Thaumatins have been identified in maize microspore embryonic suspension cultures and also in barley (Osmond et al., 2001) and wheat (YU et al., 2003) seeds. Thaumatins are PR proteins capable of binding and hydrolyzing β-1,3-glucans (Trudel et al., 1998; Grenier et al., 1999) and releasing oligosaccharides.

In carrot somatic embryogenesis, conditioned media has been reported to have a promoting effect on embryo formation (Hari, 1980; Smith and Sung, 1985). During the course of cultures, secreted proteins increase, and among them EP1 (extracellular protein 1) is released by nonembryogenic cells (van Engelen et al., 1993), whereas EP2 is secreted only by embryonic cells (Sterk et al., 1991). Another protein called EP3 was identified, corresponding to a chitinase. De Jong et al. (1992) showed that EP3 was able to rescue blocked mutant (*ts11*) embryos. The chitinases have been thought to be involved in generation of signal molecules essential for embryogenesis (de Jong et al., 1993). The responsible mechanism is largely unknown. Interestingly, EP3 was also found in developing carrot seeds. Chitinases could be expected to play a role in zygotic embryogenesis also.

The presence of EP3 may result in the generation of GlcNAc-containing molecules that have a stimulating effect on the development of somatic embryos, compared to the action of chitinases capable of hydrolyzing Nod factors (Staehelin et al., 1994). In another species, Norway spruce, endogenous Nod-factor-like signal molecules have been shown to promote embryo development (Dyachock et al., 2002). Finally, it is known that AGPs contain endochitinase cleavage sites (Van Hengel et al., 1998; Showalter, 2001). Thus, endochitinases can split AGPs and generate small molecules like oligosaccharides which may be signals to developing embryos.

SIGNALING BETWEEN EMBRYO AND ENDOSPERM STRUCTURES

In planta, the embryo develops from the fertilized egg cell (zygote), and the endosperm from the fertilized central cell. In cultures in vitro, competent cells lead to the formation of embryonic or nonembryonic structures which in turn generate embryo-like structures and endosperm-like structures in time. The question of exchanges between these different cellular structures arises, and painstaking research is needed to determine the role of secreted molecules in the two developmental systems.

The interaction between the embryo and the endosperm has been investigated mainly in terms of the nutritive aspects of endosperm as an "embryo nourishing tissue" (Schel et al., 1984; Lopes and Larkins, 1993). Indeed, endosperm seems to be absolutely required for the nourishment of young embryos (Friedman, 1995; Matthys-Rochon, 2002). Questions arise about the active molecules that appear in vivo within the microenvironment and promote normal development. The dependence of early embryonic development on the endosperm is also implied by apomictic studies (Koltunow 1993; Bicknell and Koltunow, 2004). Has the endosperm uniquely a nutritive role?

First, in somatic and haploid embryogenesis, the occurrence of two populations of multicellular structures, termed embryogenic and nonembryogenic, has been demonstrated. The idea has emerged of two types of developing structures with different potentialities: endosperm-like and embryo-like (Magnard et al., 2000; Testillano et al., 2002; Massoneau et al., 2005). Second, several molecules identified in the medium of in vitro cultures are also present in the developing seed. These molecules, AGPs (van Hengel et al., 2002; Paire et al., 2003; Borderies et al., 2004), PSK (Yang et al., 1999), chitinase, thaumatin and β-1,3-glucanase (Kragh et al., 1991; Helleboid et al., 1998, 2000; Borderies et al., 2004), are supposed to be or to generate signal molecules which direct the fate of cells (McCabe et al., 1997) and help to establish their

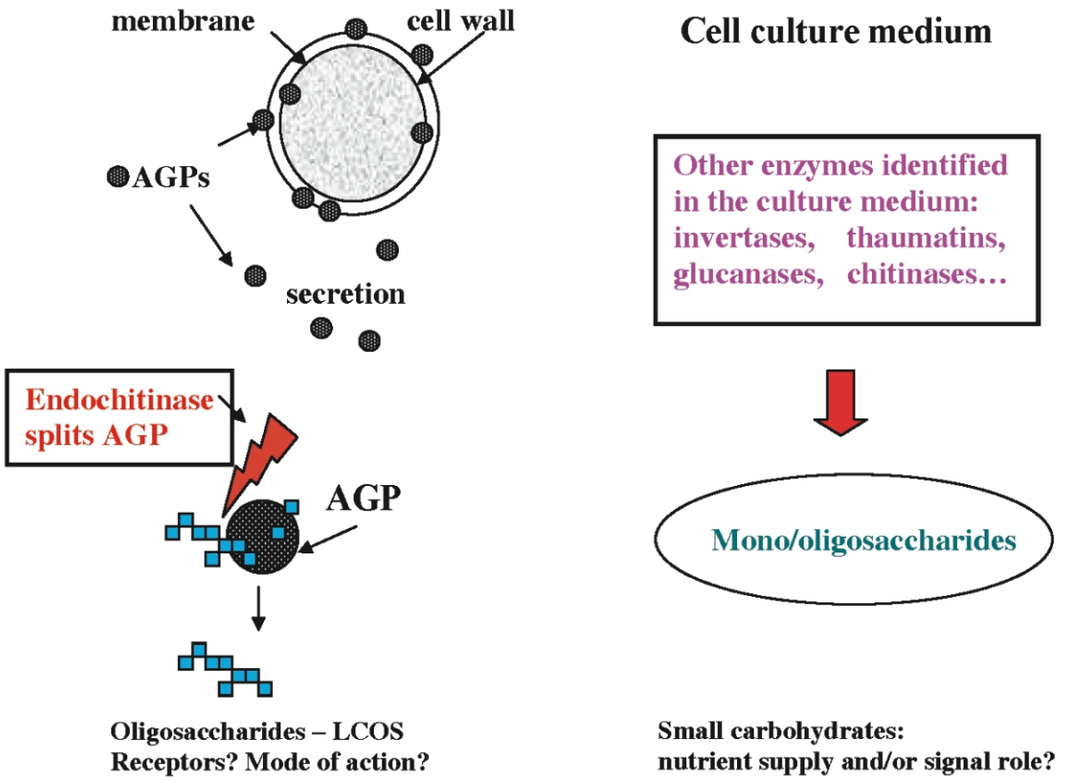

Fig. 1. Enzymes secreted by cells into culture medium and their possible role. Left: AGPs are present on the membrane and cell wall of cells in culture. They are released into the medium and may be hydrolyzed by endochitinases; oligosaccharides are generated. Right: Other identified enzymes that produce small carbohydrates (mono-, oligosaccharides) which may be either nutrients or signal molecules or both. The precise mechanisms that involve oligosaccharides in developmental processes are unknown.

embryonic competence and/or to direct the enlargement and further development of the embryo (Hanai et al., 2000).

In different species in culture, as reported above, PR proteins have been identified which may have a precise role in embryo development, associated with their function. Endo-β-1,3-glucanases are thought to produce oligosaccharides (Kragh et al., 1991; Helleboid et al., 1998, 2000); thaumatins, which bind to β-1,3-, may hydrolyze these carbohydrates (Trudel et al., 1998; Osmond et al., 2001) and release oligoglucosides (Grenier et al., 1999). Chitinases, which have been detected in different cell type cultures, can split AGPs and release oligosaccharides (Showalter, 2001; van Hengel et al., 2001). Thus, multicellular structures that secrete these different types of enzymes can produce small molecules (signal molecules?) which may intervene in embryo development. In addition, oligosaccharides present in or produced in culture media may be both signals and/or nutrients. The possible mechanisms generating small carbohydrates are showed in Figure 1. Enzymes and AGPs have been described in the surroundings of the developing embryo in planta (van Hengel et al., 1998; Massoneau et al., 2005), in endosperm (van Hengel et al., 2002), or secreted by endosperm cells in culture (Miernyk, 1987). Figure 2 summarizes the possible interactions that may occur in planta between embryo and endosperm, and compares them with what may happen between embryogenic and nonembryogenic structures in vitro.

This review and other data (Massoneau et al., 2005) suggest that in vitro developing structures may have endosperm or embryo potentialities, and may interact through signal molecules which direct embryo development. In this way, in vitro cultures mimic what exists in planta.

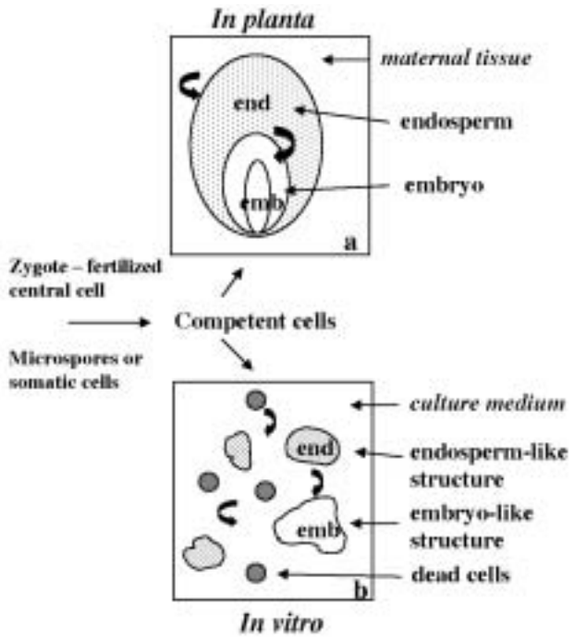

Fig. 2. Possible exchanges between in vitro or in planta embryo and endosperm structures. (**a**) After double fertilization, the zygote and the fertilized central cell become competent and develop into two structures (embryo and endosperm represented here in a grass seed). Arrows indicate interactions between embryo and endosperm and probably with maternal tissue (van Hengel et al., 2002), (**b**) Comparison with the probable situation in cell cultures (microspores or somatic cells), where competent cells generate embryonic (emb) and nonembryonic (end) structures. Secreted molecules are released and help the embryo to form (curved arrows). (Borderies et al., 2004; Massoneau et al., 2005).

CONCLUSION

This review does not cover all the recent progress in the study of plant signal molecules (Minorsky, 2003) and their influence on embryological processes.

During the last decade, biochemical studies have demonstrated that during embryo formation in plants, different types of molecules, mainly oligosaccharides, enzymes and arabinogalactan proteins (this review), are synthesized and secreted into the cell environment, and that they play a developmental role. Biochemical studies will be completed with investigations of genes and mutant strategies. Thus, the mechanisms involved in embryo formation are soon likely to be elucidated more completely. Thus, the challenge in the coming years will be (1) to determine the potential function of the stimulating molecules, and (2) to identify the ligands and receptors that take part in the construction of the embryo by stimulating metabolic pathways which permit the differentiation and growth of a new plantlet.

The categories of molecules that have been cited are embryo-stimulating factors, but the list is not complete (Weber, 2002). Fatty acids are new candidates in the search for signal molecules acting in plant development and possibly on embryo formation.

This review raises a crucial question: do the identified molecules present and important in development in vitro also function during the development of zygotic embryos? At the moment, the factors that act on cells in vitro to make them behave like zygotes are unknown, and the same applies to the specificity of substances emanating from the endosperm. It seems likely that the endosperm has two roles: to give nutrients to the embryo and to direct its fate.

It is fascinating to think that cells in vitro seem to invent processes similar to those that occur in planta. This makes study and comparison of the two types of development a powerful tool. Although in recent years there has been much progress in understanding how a plant embryo forms, there is still a long way to go.

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