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GENE EXPRESSION DURING INDIRECT SOMATIC EMBRYOGENESIS OF PLANTS

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Somatic embryogenesis is the process by which somatic cells are induced into an embryogenic state, followed by differentiation into embryos. Somatic embryogenesis, in addition to being a method of propagation, can serve as an experimental tool for research into plant embryo development. This is a review of the current literature on *in vitro* plant somatic embryogenesis and the molecular advances made to identify genes expressed during the various stages of this process. Some factors hindering the elucidation of the molecular mechanisms underlying somatic embryogenesis are discussed.

L'embryogenèse somatique est le processus par lequel les cellules somatiques passent à l'état embryogène et se différencient en embryons. En plus de constituer une méthode de propagation, elle peut servir d'outil expérimental de recherche pour développer des embryons de plantes. Le présent document est une revue de la documentation sur l'embryogenèse somatique végétale *in vitro* et sur les progrès réalisés à l'échelle moléculaire pour identifier les gènes exprimés au cours des divers stades du processus. On examine aussi certains facteurs qui rendent difficile l'élucidation des mécanismes moléculaires de l'embryogenèse somatique.

Introduction

Plants are multicellular organisms, composed of highly organized tissues and organs. Plant development, like that of all multicellular organisms, involves the differentiation of cells such that they "acquire distinct metabolic, structural and functional properties" (Taiz & Zeiger 1998). Plant cells differentiate into either sexually reproductive cells or somatic (asexual) cells. Sexually reproductive cells are responsible for the production of a new generation; the fusion of male and female gametes involves the recombination of genetic information through meiosis. This genetic mixing results in offspring that are genetically different from the parent plants. Genetic diversity provides plants with a selective advantage helping to ensure survival of the species during environmental change.

Somatic or asexual cells have two distinct functions: 1) vegetative growth and 2) asexual reproduction. Vegetative growth involves the increase in plant cell size and number. During their lifespan, plants must continuously generate new tissues and organs, for example, to repair the damage caused by herbaceous animals. Asexual reproduction involves the production of plants genetically identical to the single parent plant, a process also known as cloning. Asexual reproduction allows plants to reproduce when the costs of sexual reproduction are too high, environmental conditions are not suitable for sexual reproduction or in cases where individuals may be isolated, in terms of distance, from other individuals. Plants are capable of asexual reproduction and regeneration of lost parts as a result of the totipotent nature of their

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cells. Many plant cells are totipotent, meaning that under appropriate conditions, they are able to differentiate into "the entire spectrum of cell types" found within the plant (Weigel & Jürgens 2002).

For most animal cells, the differentiation process is irreversible while plant cells have the unique ability to *de*differentiate. Upon dedifferentiating, plants can access the genetic information for the entire genome and therefore regain the undifferentiated or embryonic state. Given the sessile state of plants, this ability to "adapt their programme of differentiation and growth" is an important survival mechanism (Roberts et al. 2002). This survival mechanism is evident in a plants' ability to grow back lost parts consumed by animals.

The abilities of plants to reproduce asexually and plant cells to dedifferentiate have been used in plant propagation through plant tissue culture. Plant tissue culture is a broad term referring to the growth of plant cells, tissues or organs under sterile conditions in culture (*in vitro*). One process carried out through plant tissue culture is that of somatic embryogenesis (SE). SE is the development of bipolar embryos from somatic cells and tissues; bipolar meaning they possess both a shoot and a radical end (Sharp et al. 1980). Somatic cells are induced to dedifferentiate and are then reprogrammed to develop into bipolar embryos. The embryos produced via SE, referred to as somatic embryos, are capable of developing into complete plants. The embryos are produced asexually; therefore the resulting plants are genetically identical to the tissue from which the embryos were derived. Although somatic embryogenesis has been reported to occur naturally in species of *Bryophyllum* (Yarbrough 1932) and *Malaxis* (Taylor 1967), it is best known as a pathway used in plant tissue culture to propagate plants. The first report of somatic embryogenesis *in vitro* was in carrot (*Daucus carota* L.) (Steward et al. 1958). The fact that the embryos are bipolar is ideal for propagation in that it allows for simultaneous root and shoot formation. In addition to being a method of propagation, SE is a valuable tool for studying zygotic plant embryo development, which is a difficult task given the intact nature of plant seeds.

Somatic Embryogenesis

General

There are two types of somatic embryogenesis: direct and indirect. Direct SE refers to the process of inducing somatic embryos or embryogenic tissue directly from the differentiated tissue. Zygotic embryos are one of the most successful sources of initiating direct SE (Merkle et al. 1990). Quite often however, the objective of SE is to clone a mature plant after its characteristics and performance have been evaluated and found to be desirable. This objective is especially true of plants whose economic market is driven by consumers' demands for specific floral forms and horticultural traits such as disease resistance (Marchant et al. 1996). Zygotic embryos cannot be used to clone a mature plant since they are produced sexually and therefore are genetically different from the plant that produced them. To clone a mature plant, tissues from the plant itself must be used to induce somatic embryogenesis. Somatic tissues exposed to a high concentration of the plant growth regulator, 2,4-dichlorophenoxyacetic acid (2,4-D) have been reported to undergo direct somatic embryogeneis (Kitamiya et al. 2000). The use of somatic tissues for inducing direct SE is hampered by the fact that not all species are capable of this and that high concentrations of 2,4-D cause genetic mutations that encumber the objective of clonal propagation (Swartz 1991).

Most somatic tissues require a "genetic activation" in order to enter an embryogenic state and be capable of producing embryos. Indirect SE involves an intermediate stage

prior to somatic embryo or embryogenic tissue formation. Indirect SE has been induced using somatic tissue in several species, such as African violet (*Saintpaulia ionantha* Wendl.) (Mithila et al. 2003), grape (*Vitis vinifera* L.) (Das et al. 2002), rose (*Rosa* sp.) (Ibrahim & Debergh 2001, Kintzios et al. 1999, de Wit et al. 1990), sunflower (*Helianthus maximiliani* Schrader) (Vasic et al. 2001), and Siberian ginseng (*Eleutherococcus senticosus*) (Choi et al. 1999). The goal is to induce the source tissue (explant) to dedifferentiate and form callus; a mass of unorganized cells (Figure 1). The callus is not yet committed to differentiate into any organized structure and must be further "genetically activated" to produce embryos. It has been reported that the initiation of embryogenic tissue from differentiated explants often involves "extensive proliferation through an unorganized callus cycle" (Merkle et al. 1990). Factors reportedly used to activate callus growth include growth regulators, such as auxins (Murashige & Tisserat 1977), stress such as starvation of the tissue (Lee et al. 2001), and heat shock. Frequently, exposure to a low concentration of 2,4-D followed by transfer to an even lower concentration of 2,4-D or growth regulator-free medium has been successful in inducing SE in a wide range of plant species, as in Siberian ginseng (*Eleutherococcus senticosus*) (Choi et al. 1999), rose (*Rosa* sp.) (de Wit et al. 1990), oat (*Avena sativa)* (Chen et al. 1994), and carrot (*Daucus carota)* (Toonen et al. 1994). The removal of 2,4-D is not believed to be responsible for initiating SE however it allows the progression of the pre-embryonic cells to the advanced stages of somatic embryo development.

The structural characteristics of embryogenic cells are typical among many species; consisting of small cells with dense cytoplasmic contents, small vacuoles and large nuclei with very distinct enlarged nucleoli (Williams & Maheswaran 1986). The external morphology of both callus and embryogenic tissue varies greatly amongst and within plant species (Rout et al. 1999). Toonen et al. (1994) reported high morphological variability among single suspension cells of carrot (*Daucus carota*) that were competent to become embryogenic. This variability makes it difficult to distinguish or predict cells with embryogenic competence even within an individual. Positive identification of embryogenic tissue is sometimes only known by the presence of nodular structures. This nodular tissue is often referred to as proembryogenic masses (PEMs) (von Arnold et al. 2002). The nodular structures are actually early globular stage embryos that precede the later stages of dicotymous embryo development, i.e., heart-shaped, torpedo-shaped and cotyledonary embryos (Figure 2).

Once somatic embryos reach the cotyledonary stage, they must go through sequential stages of maturation, a desiccation or cold period, germination, acclimatization and transfer to in the *ex vitro* environment. There is much diversity among and within plant species in terms of the stages and time lines of SE (Das et al. 2002, Ibrahim & Debergh 2001, Vasic et al. 2001, Kintzios et al. 1999, de Wit et al. 1990). Despite this diversity, it is known that the various stages of SE involve the "commitment of specific cells to a sequential pattern of selective gene expression" (Zimmerman 1993, Giroux et al. 1997).

Gene expression during somatic embryogenesis

Each stage of somatic embryogenesis involves the activation and deactivation of genes. Certain plant developmental processes are likely the result of an array of interacting genes, which require the expression of proceeding genes (Torres-Ruiz et al. 1996). The isolation of embryo-specific genes and the characterization of their roles during embryo development are fundamental in the overall understanding of the molecular processes regulating embryogenesis (Magioli et al. 2001). Understanding

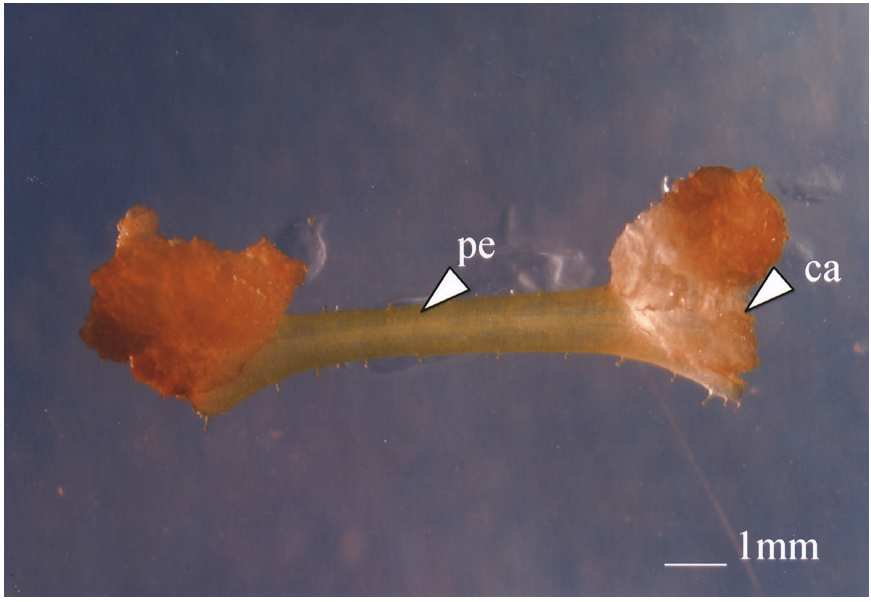

Fig 1 Callus (ca) developed on the excised ends of a rose petiole explant (pe) after two weeks growth on medium containing 5μM 2,4-D.

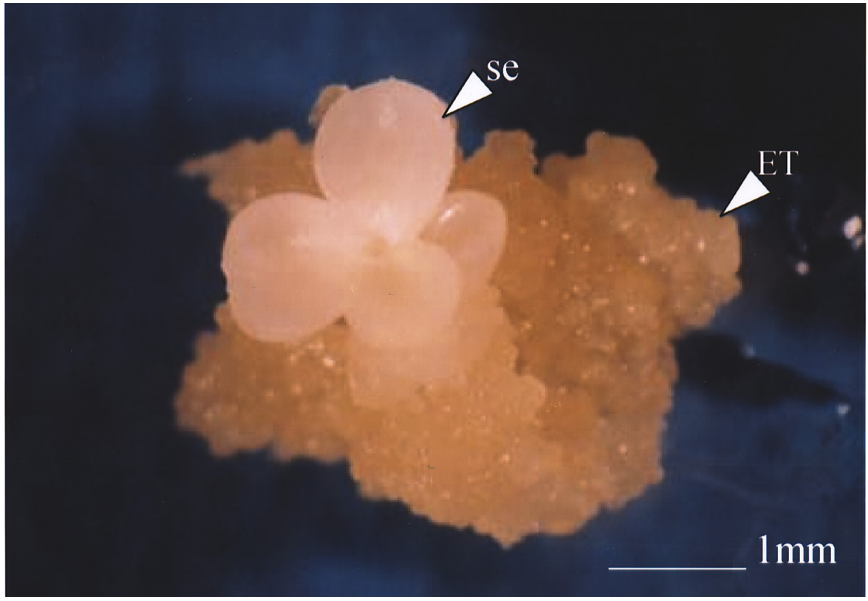

Fig 2 Somatic embryo (se) growing on embryogenic tissue (ET) after transfer of tissue to growth regulator-free medium.

the genetic control of development is one of the most fundamental questions in biology and remains one of the main research areas of molecular biology today.

The first step in indirect somatic embryogenesis is to dedifferentiate somatic cells to form callus. As previously mentioned, exposure to the plant growth regulator 2,4-D is one means of achieving this. Reprogrammed gene expression is evident by the synthesis of new messenger ribonucleic acid (mRNA) after exposing cells growing *in vitro* to 2,4-D (Hagen et al. 1984, Theologis 1986, Van der Zaal et al. 1987). The exact stages and mechanisms involved in the transition of callus cells into embryogenic cells are not known. Quite often the callus induction stage and its subsequent transition to an embryonic state is collectively referred to as early somatic embryogenesis (Kairong et al. 1999, Sato et al. 1995, Momiyama et al. 1995).

Recent studies have attempted to identify differences in gene expression between callus and embryogenic tissue. Duncan and associates (2003) found that tissue with high levels of *globulin-1* (*Glb1*) - protein encoded polypeptides is embryogenically competent. In non-embryogenic tissues, very low concentrations of *Glb1* were detected. The *Glb1* protein is known to be synthesized in zygotic embryos shortly after pollination.

In carrot (*Daucus carota*), the Somatic Embryogenesis Receptor-like Kinase (SERK) gene was found to be a useful marker of single cells possessing competency to form somatic embryos (Schmidt et al. 1997). SERK gene expression was detected in a cohort of slightly elongated and vacuolated cells throughout the developmental stages prior to reaching the globular stage embryos. Somleva and associates (2000) also identified the SERK gene during the induction of "embryogenic cell formation" in single cells of *Dactylis glomerata*.

HBK2, a new gene belonging to class I of the KNOTTED1-like homeobox (KNOX) genes was expressed in Norway spruce (*Picea abies* (L.) Karst.) during proembryogenic masses through to late stage somatic embryo development (Hjortswang et al. 2002). Homeobox genes control cell specification and pattern formation during plant development. The HBK2 gene was expressed only in embryogenic cell lines that resulted in somatic embryo production and not in cell lines that failed to produce embryos. Other related KNOX genes have been reported to be differentially expressed during both zygotic and somatic embryogenesis of maize (*Zea mays* L.) (Zhang et al. 2002).

Kairong and associates (1999) obtained three complementary deoxyribonucleic acids (cDNAs) from early somatic embryogenesis of *Lycium barbarum,* which were not observed in calli. The cDNAs were produced after the transfer of the tissue from 2,4-D to an auxin-free medium. Although the roles of these cDNAs were not discussed, the results of this study support the notion that callus induction and its transition into an embryogenic state are two distinct processes involving the expression of distinct genes. Likewise, Giroux & Pauls (1997) identified three cDNAs transcripts (*ASET1, ASET2,* and *ASET3)* present in embryogenic tissues of alfalfa (*Medicago sativa* L.) but absent in petioles, mature embryos or non-embryogenic tissue.

Several genes expressed during the various stages of somatic embryo development have been identified. New gene products synthesized in plants upon the removal of auxin from the medium were reported by Borkind and associates (1988).. These gene products are required for the transition from globular stage embryos to the heart-shaped stage.

The *CEM6* gene coding for a protein expressed during early embryo development with the highest levels occurring at the early globular stage (Sato et al. 1995) reportedly began to decrease at the later torpedo-shaped stage (Komamine et al. 1999). Magioli and associates (2001) reported the expression of the glycine-rich *Atgrp-5* gene during

early embryo development of *Arabidopsis thaliana* and eggplant (*Solanum melongena* L.). *Atgrp-5* was detected in globular and torpedo stage embryos but eventually turned off in later stage cotyledonary embryos.

Several genes expressed during somatic embryo development appear to have characteristics of a class of proteins called Late Embryogenesis Abundant (LEA) proteins (Sunderlíková & Wilhelm 2002, Dure et al. 1981, 1989, Galau et al. 1986). Most of the LEA transcripts increase significantly in somatic embryos at the heart stage (Choi et al. 1987, Wilde et al. 1988, Franz et al.1989, Wurtele et al. 1993). The LEA genes appear to be induced by the application of abscisic acid (ABA) (Hatzopoulos et al. 1990, Goupil et al. 1992) as well as other factors such as water stress (Han et al. 1997). ABA is a growth regulator produced naturally by plants during zygotic embryogenesis and is commonly employed during the maturation stage of SE (von Arnold et al. 2002). Dong & Dunstan (1997) identified five ABA-responsive cDNAs from white spruce (*Picea glauca* L.). Three of the cDNAs (PgEMB12, 14 and 15) were speculated to encode LEA proteins while the other two (PgEMB5 and 23) were not similar to any known DNA or protein sequences. Sunderlíková & Wilhelm (2002) reported the accumulation of mRNAs during the maturation of oak (*Quercus robur* L.) somatic embryos that were similar to that of the LEA proteins observed during late cotyledonary embryos development.

Early molecular studies evaluating gene expression in SE focused mainly on the developmental stages of somatic embryos with little attention being paid to the initiation of somatic embryogenesis (Zimmermann 1993). The fact that somatic embryo development is readily divided into distinct stages based on morphological characteristics (globular, heart-shaped, torpedo-shaped and cotyledonary) makes it an easier task to approach than that of the less well-defined stages of early somatic embryogenesis. Although there are both molecular and morphological differences between non-embryogenic and embryogenic tissue, the transition from one stage to the next is still a very grey area. It is difficult to identify differences in gene expression between stages when the stages themselves are not well defined.

Conclusions

Somatic embryogenesis can be induced by several different factors; plant growth regulators, stress, and heat shock. The fact that there is more than one SE inducer suggests that the transition from a somatic cell to an embryogenic cell may have several different molecular routes, further complicating the task of understanding the molecular basis.

A greater emphasis on characterizing and defining the stages involved from dedifferentiation through to the transition of callus into an embryogenic state is required in order to elucidate the processes involved in indirect SE. The understanding of the stages involved in early SE and their underlying molecular mechanisms are linked; advances in one area will facilitate the understanding of the other.

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