Endogenous Factors that Regulate Plant Embryogenesis: Recent Advances

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ABSTRACT

In seed plants, embryogenesis is an important process to produce a new generation. It comprises three steps: establishment of organization as an embryo, accumulation of storage substances in the embryo, and acquisition of desiccation tolerance and seed dormancy. These steps are accurately regulated by many factors, including phytohormones, proteins, transcription factors, and other substances related to embryogenesis. The embryogenesis mechanism has been analyzed through biochemical, biological, and molecular approaches using embryo-defective mutants or somatic embryogenesis whose traits are similar to zygotic embryogenesis, both morphologically and physiologically. Appropriate auxin transport plays an important role in the formation of cotyledon and meristem during early embryogenesis. Some transcription factors (LEC1, AB13, LEC2, and FUS3) that have been isolated from embryo-defective mutants are characterized as embryo-related genes. Among them, some transcription factors are related to phytohormone signaling. ABI3 and ABBA regulate the expression of the LEA gene, whose proteins are accumulated during late embryogenesis. Also, LEC2 and FUS3 negatively regulate bioactive GA synthesis. On the other hand, some regulatory factors have been isolated and identified from culture medium during somatic embryogenesis. The factors are low molecular substances such as the phenolic compounds (4HBA, VBE and 4PMP) that inhibit somatic embryogenesis, or the peptidyl growth factor, PSK, which stimulates somatic embryogenesis. Here, we review recent findings of various factors regulating plant embryogenesis.

Keywords: phenolic compounds, phytohormone, seed plants, somatic embryo, transcription factor, zygotic embryo

Abbreviations: ABA, abscisic acid; AB13, ABA-insensitive3; ABRE, ABA responsive element; AGL15, AGAMOUS-like 15; 2,4-D, 2,4-dichlorophenoxyacetic acid; EC, embryonic cells; FUS3, FUSCA3; GA, gibberellic acid; 4HBA, 4-hydroxybenzyl alcohol; LEA proteins, late-embryogenesis abundant proteins; LEC, LEAFY COTYLEDON; PIN, PIN-FORMED; PLT, PLETHORA; 4PMP, 4-[phenylmethoxy(methyl)] phenol; PSK, phytosulfokine; SAM, shoot apical meristem; VBE, vanillyl benzyl ether; VPI, VIVIPAROUS 1

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INTRODUCTION

In seed plants, embryogenesis is an important morphogenesis involving drastic changes by which an individual is born from a fertilized egg. A zygote divides transversely and asymmetrically to form a small apical cell and a large basal cell. The apical cell develops to the embryo proper, and the basal cell develops to the suspensor, which remains attached to the mother tissue and provides nutrients and growth regulators for development of the embryo proper. Development of the embryo proper is distinguishable as three steps: establishment of organization as an embryo, accumulation of storage substances in the embryo, and acquisition of desiccation tolerance and seed dormancy.
LEC1 in developing embryos, and the L1L ectopic expression (Kwong et al. 2003). Between the LEC1 and L1L, the B domain is highly conserved and Asp-55 is most important for these protein functions in developing embryos (Lee et al. 2003).

**FUSCA 3 (FUS3)**

FUS3 encodes a B3 domain-containing protein, whose gene expression is observed in developing embryos from a very early stage to immediately before germination (Luerßen et al. 1998). Embryos of the fusca3 mutant (fus3) produce trichomes on the cotyledons and exhibit increased accumulations of anthocyanin and decreased accumulations of seed storage proteins compared to wild-type embryos (Bäumlein et al. 1994; Keith et al. 1994). The FUS3 protein binds directly to RY motif, which is conserved in the promoter of many seed-specific genes and which regulates the expression of these genes during embryogenesis (Kroj et al. 2003; Mönke et al. 2004). Induction of FUS3 gene expressions in the L1 layer of the shoot apical meristem (SAM) using AtML1 promoter produces cotyledon-like organs in the transgenic Arabidopsis SAM (Gazzarrini et al. 2004).

**LEAFY COTYLEDON 2 (LEC2)**

LEC2 encodes B3-domain-containing protein, which is related closely to FUS3 (Stone et al. 2001). Embryos of the lec2 produce trichomes on the cotyledons and display abnormal suspensor morphology. Expression of LEC2 is siliquose-specific, and ectopic expression of the LEC2 gene induces the formation of somatic embryo-like structures; it often confers embryonic characteristics to seedlings. LEC2 protein binds to RY motif upstream of target genes (Braybrook et al. 2006). Expression of LEC2 gene in leaves regulates the gene expression of ABI3, FUS3, and LEC1 genes and seed-specific lipids accumulation (Santos-Mendoza et al. 2005). In lec2 mutant, the expression of FUS3: GUS and ABI3:GUS is reduced. The seed phenotype is partially restored by introgression of 35S:FUS or 35S:ABI3 genes (To et al. 2006). These results indicate that LEC2 functions in the upstream of ABI3 and FUS3 in some cases.

**ABA-Insensitive 3 (ABI3) / Viviparous 1 (VP1)**

Arabidopsis abi3 and maize vp1 are seed-specific ABA-insensitive mutants. Seeds of these mutants undergo viviparous germination, have no seed dormancy, acquire no desiccation tolerance, and accumulate few seed storage proteins. ABI3/VP1 contains some conserved domains (B1, B2, and B3), of which B2 and B3 might be involved in seed-specific gene expression. Expression of ABI3/VP1 is observed mainly in embryos; ABI3/VP1 expression during zy-
gotic embryogenesis begins at a very early stage and is detectable continuously until the late stage of embryogenesis. The expression of ABI3 is also regulated by LEC1, LEC2, FUS3 and ABI3 itself (To et al. 2006).

On the other hand, ABI3 protein controls ABA-induced Late-Embryogenesis Abundant (LEA) gene during the late stage of embryogenesis expression (Parry et al. 1994). Analyses of the mechanisms regulating the expression of seed-specific ABA-inducible genes (Em and fcm) suggest that the B2 domain of ABI3/VPP regulates the expression of ABA-inducible genes via the cis-regulatory ABA responsive element (ABRE), which resembles the G-box element (Marcotte et al. 1989; Hattori et al. 1995). In the regulatory scheme, ABI3/VPP does not bind to ABRE directly, but might bind via formation of a complex with bZIP proteins (Nakagawa et al. 1996; Lopez-Molina et al. 2002; Lara et al. 2003). These results suggest that ABI3 is an important factor in various ABA responses during embryogenesis and germination.

Function of the transcription factors in somatic embryogenesis

Expression of LEC1 and LEC1-homologs is observed with the same pattern during somatic embryogenesis in Arabidopsis, maize, and carrot (Ikeda-Iwai et al. 2002; Zhang et al. 2002; Yazawa et al. 2004). In-situ hybridization analysis reveals that the expression patterns of ZmLEC1 and C-LEC1 are similar in zygotic and somatic embryos (Zhang et al. 2002; Yazawa et al. 2003). Expression of FUS3 occurs in somatic embryos of Arabidopsis (Ikeda-Iwai et al. 2002, 2003), but expression of LEC2 during somatic embryogenesis remains to be examined. In lec1, fus3, and lec2 single and double mutants, the frequency of somatic embryo formation is very low (Gaj et al. 2005), which suggests that these factors have an important function in formation of plant somatic embryo.

ABI3 gene expression is also observed in somatic embryos of Arabidopsis (Ikeda-Iwai et al. 2002, 2003) and carrot (Shiota et al. 1998). The data indicate that ABI3 also regulates ABA signal transduction in somatic embryos. However, somatic embryos might be formed. Ikeda-Iwai et al. (2002, 2003) showed ABI3 gene expression in somatic embryos and embryonic cultures of Arabidopsis. However, ab3-6, a null mutant of ABI3, suggests that ABI3 is not an essential factor for somatic embryo formation (Umehara and Kamada 2004).

LOW MOLECULAR WEIGHT SUBSTANCES

Auxin

Auxin is an important phytohormone in many processes during plant development. In early embryogenesis, auxin transport and distribution mainly affect the embryo axis formation. Here, we review PIN proteins, which serve an important role in auxin polar transport, although many reports describe the effects of auxin in embryogenesis. Auxin polar transport in embryogenesis was reported in early studies that they used as the plant material. To induce somatic embryogenesis, not only complete somatic embryos but also adventitious and fused shoots are often formed (Bassuner et al. 2007). In abnormal shoots, PIN4 expression is not observed at the base of shoots. For establishment of a root meristem in somatic embryos, maintenance of appropriate auxin levels through PIN proteins is required during the course of their development.

Furthermore, PIN proteins interact with PLETHORA (PLT) genes, major determinants for root stem cell specification (Bilou et al. 2005). Also, PIN proteins restrict PLT expression in the basal region of the embryo proper to initiate root primordium formation. Then PLT genes maintain PIN transcription, which stabilizes the position of the distal stem cell niche.

In addition to PIN proteins, MONOPTEROS, BODENLOS, and some proteins are involved in auxin transport or response. Interactions between these proteins and PIN proteins should also be considered for auxin transport and response during embryogenesis.

Gibberellic acid (GA)

GA is a tetracyclic diterpenoid that is an essential endogenous regulator of plant growth and development. Earlier studies demonstrated that endogenous GA levels in the suspensor are higher in that than in the embryo proper, and that GA might play an important role in early embryo development for Phaseolus (Alpi et al. 1975), Tropaeolum, and Cytisus (Picciarelli et al. 1984). In pea, GA produced in the embryo is necessary for normal seed growth and survival (Swain et al. 1997). In microspore-derived embryos of Brassica, auxin is required for elongation of the cell and embryo axis (Hays et al. 2002).

In 2003, the opposite results were proposed by Tokui et al. and Mitsuhashi et al. on the effect of gibberellin in carrot somatic embryogenesis. Tokui et al. (2003) demonstrated that GA inhibits somatic embryogenesis, but Mitsuhashi et al. (2003) showed that GA is required for somatic embryogenesis. We would like to specifically address the explants used in the experiments. To induce somatic embryogenesis, the former used hypocotyls (somatic cells) as the explant, the latter used embryogenic cells (EC) which had acquired embryogenic potential.

On the other hand, postembryonic expression of LEC2 induces the formation of somatic embryos on the cotyledon (Stone et al. 2001). The gene expression of AtGA3ox2, which encodes the key enzyme AtGA3ox2, which catalyzes the ultimate step of the active GA synthesis, is negatively regulated by transcriptional factor, LEC2 and FUS3 in Arabidopsis (Curaba et al. 2004; Gazzarrini et al. 2004). FUS3 represses AtGA3ox2 expression mainly in epidermal cells of the embryo axis. AtGA2ox6, which encodes AtGA2ox6, which converts active GA to inactive GA, is regulated by AGL15, a member of the MADS domain family of DNA binding transcriptional regulators (Wang et al. 2004). Although somatic embryos are formed when AGL15 constitutively expresses, the frequency of somatic embryo forma-
Abscisic acid (ABA)

In plant embryogenesis, ABA is synthesized at the late stage of embryogenesis and controls the acquisition of desiccation tolerance and seed dormancy. In Arabidopsis, ABA-deficient mutants, embryogenesis progresses normally but the mutants have no desiccation tolerance or seed dormancy. It is not considered that ABA is directly related to zygotic embryo development.

However, somatic embryogenesis can be induced by treatment with various stresses such as high osmotic stress and high temperature under a 2,4-D-free condition in carrot (Kobayashi et al. 2006). After various stresses, ABA is known to increase. Therefore, it has been proposed that increased endogenous ABA levels might induce somatic embryogenesis. Some reports suggest that ABA is involved in somatic embryogenesis. Treatment with 10^{-7} M ABA induces somatic embryo formation in carrot apical tip explants (Nishiwaki et al. 2000). Somatic embryos (primary embryos) form from seed coats without auxin treatment (Ogata et al. 2005). Subsequently, many secondary embryos are produced on the hypocotyl and root region of the primary embryos. The primary embryos contain higher levels of ABA than secondary embryos. Formation of secondary embryos is suppressed by treatment with fluridone, an ABA-synthesis inhibitor. Kikuchi et al. (2006) reported that ABA is required for acquisition of embryogenic competence of somatic cells using carrot stress-inducible somatic embryogenesis. These results indicate the possibility of mutually competitive roles of ABA and GA in embryogenesis.

On the other hand, different results are shown in Arabidopsis. Using stress-inducible somatic embryogenesis in Arabidopsis (Ikeda-Iwai et al. 2003), the effect of ABA was investigated (Umehara and Kamada 2004). Somatic embryogenesis is induced by treatment with fluridone, and is recovered by addition of ABA. However, somatic embryos are induced from ABA deficient mutants and transformants; they are rather inhibited by addition of ABA (Umehara and Kamada 2004). Gaj et al. (2006) also shows that the efficiency of somatic embryo induction in ABA deficient mutants is comparable to that of the wild type in Arabidopsis somatic embryogenesis. In Arabidopsis, addition of 2,4-D is required for stress-inducible somatic embryogenesis, different from that of carrot. Although endogenous ABA content increases during seed maturation, no somatic embryo forms within the seed. Other factors (s) might be considered to act during somatic embryogenesis to connect these inconsistent results.

Phytosulfokine

Somatic embryogenesis depends on several modulatory substances, some of which accumulate in culture medium. From culture medium of Asparagus officinalis L., Matsubayashi and Sakagami (1996) isolated phytosulfokine (PSK), the sulfated pentapeptide H-Tyr(SO_3H)-Ile-Tyr(SO_3H)-Thr-Gln-OH, which stimulates mitogenic activity in mesophyll cells of Asparagus. The effects of PSK on cell division and morphogenesis have also been examined in various species of angiosperms, including Oryza sativa, Arabidopsis thaliana, Zea mays, and asparagus (Matsubayashi et al., 2003). These facts suggest that PSK is a common peptide substance in seed plants and plays a basic role in plant growth and development.

In carrot, exogenously applied PSK stimulates somatic embryogenesis by activating cell division of embryogenic cells (EC; Kobayashi et al. 1999, Hanai et al. 2000). The stimulatory effect of PSK in somatic embryogenesis has been confirmed in gymnosperms: Cryptomeria japonica (Igasaki et al. 2003) and Larix leptolepis (Umehara et al. 2005b).

PSK stimulates cell division under the presence of auxin and cytokinin in asparagus (Matsubayashi et al. 1999) and under the presence of auxin in carrot (Eun et al. 2003). Matsubayashi et al. (2002) identified PSK receptor gene, which encodes leucine-rich repeat, a single transmembrane domain, and a cytoplasmic kinase domain. PSK binds the membrane-localized receptor PSKR1, which is a leucine-rich repeat receptor kinase (Matsubayashi et al. 2002). A loss-of-function mutant of PSK receptor gene, pskr1-1, exhibits morphologically normal growth until 3 weeks after germination (Matsubayashi et al. 2006). Therefore, PSK might be a supporting factor, rather than a critical factor, for cell proliferation and differentiation in embryogenesis.

Phenolic compounds

In carrot, EC, which have embryonic competence, are induced when explants are cultured on medium containing 2,4-D (Kamada and Harada 1979). After transfer of EC to medium without 2,4-D, somatic embryos form from EC. However, when EC are cultured at high cell density, somatic embryogenesis is strongly inhibited, even with the use of 2,4-D (Fridborg et al. 1978). The efficiency of somatic embryo formation can be improved by adding activated charcoal, which absorbs some inhibitory factors. Various phenolic compounds are accumulated in culture medium when charcoal is not present. For this reason, phenolic compounds have long been thought to inhibit somatic embryogenesis. An inhibitory factor isolated from the carrot culture medium was first characterized as a 4-hydroxybenzyl alcohol (4HBA; Kobayashi et al. 2000b). 4HBA specifically inhibits rapid cell division during the early globular stage of somatic embryogenesis (Kobayashi et al. 2000b) and accumulates in the medium during the early days of culture (Kobayashi et al. 2001). The production and inhibitory effects of 4HBA have been found not only in somatic embryogenesis but also in zygotic embryo formation of carrot (Kobayashi et al. 2003).
of Japanese larch, some conditioning factors suppress somatic embryogenesis by blocking the development of the suspensor in high-cell-density culture (Umehara et al. 2005a). Somatic embryogenesis occurs under high-cell-density conditions in the medium with activated charcoal. An inhibitory factor that accumulates in the medium can be purified; vanillyl benzy l ether (VBE) has been identified from such a culture medium (Umehara et al. 2005a). VBE is accumulated in high concentrations (greater than 10 μg) in high-cell-density cultures. However, this inhibitory effect is smaller than that of the addition of medium cultured at high cell density. Therefore, VBE is a main inhibitory conditioning factor that regulates suspensor development, but other inhibitory factors remain to be discovered. Recently, another complementary inhibitory factor was identified as 4-[(phenylmethoxy)methyl] phenol (4MP), which had a similar chemical structure to VBE (Umehara et al. 2005). However, the physiological meaning of these phenolic compounds in seed development remains unknown. At least, these phenolic compounds should be eliminated to improve the conifer tissue culture system.

**PERSPECTIVES**

Embryogenesis is a complex process that is regulated by various factors including phytohormones, proteins, and transcription factors. Many chemical substances act in gene expression as signals, and the correct expression is required for normal and rapid development of the embryos. Each factor controlling embryogenesis has been investigated using somatic and zygotic embryos in various plants. Recently, novel factors affecting plant embryogenesis have been identified; cross-linking between phytohormone and transcription factors is likely to display a part of embryogenesis. However, the mechanism of plant embryogenesis remains partially unclear. Recently, results of epigenetic approaches suggest that DNA methylation and chromatin modification are also important factors for plant embryogenesis (Reyes 2006; Xiao et al. 2006). Future studies will clarify and connect the interactions of these factors, thereby revealing the entire embryogenesis regulatory mechanism.

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JAPANESE ABSTRACT

種子植物において、胚発生は次世代を作る重要な形態能である。胚は、それらとしての基本構造の確立、貯藏タンパク質の蓄積、乾燥耐性獲得と種子休眠という大きく3つの過程が含まれ、種子植物ホルモン、タンパク質、転写因子など多様な物質によって精密に制御されている。これまで、異なる胚発生を示す変異体や種子胚に似た変動を示す不定形系を用いて、胚発生のメカニズムについて調べられてきた。例えば、種子植物ホルモンでは、オーキシンの極性輸送が初期胚の子葉の分化や分裂組織の発達に重要である。また、胚発生に関する転写因子（LEC1, ABI3, LEC2, FUS3）のうち、LEC2, FUS3はジェレインの合成を制限し、ABI3はABAとともに胚発生中に作られるLEAタンパク質の合成を制御している。一方、不定形の培養培地から胚発生を制御している物質に新たな単離されている。この観察では、植物の胚発生を制御する因子の最近の見解について紹介する。