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Review

Development of the embryo proper and the suspensor during plant embryogenesis‡

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Abstract Seed plant zygotes differentiate into two components, the embryo proper and the suspensor. Previous studies have led to the generally accepted view that development of the embryo proper is regulated by the suspensor connecting the embryo proper to donor tissue. However, biochemical, biological, and molecular analyses of embryogenesis are difficult, since zygotic embryos in higher plants are deeply embedded in mother tissues. To find a way out of the difficulties, some embryo-defective mutants of *Arabidopsis* have been used to discuss embryogenesis and suspensor function. On the other hand, somatic embryogenesis is widely used as a model system for studying the process of zygotic embryo formation. Because somatic embryo of gymnosperms has a well-developed suspensor, it has been successfully used to observe the suspensor directly and to identify factors modulating the interaction between the embryo proper and the suspensor. Various stimulatory and inhibitory factors are correlated with the interaction. Here, we review the results of studies employing *Arabidopsis* mutants and some gymnosperm tissue culture, and we discuss the possibility of using somatic embryogenesis as a new model for studies of suspensor biology.

Key words: Embryogenesis, embryo proper, gymnosperm tissue culture, suspensor.

A flowering plant zygote usually divides transversely and asymmetrically to form a terminal cell that gives rise to the embryo proper and a basal cell that divides rapidly to form a structure known as the suspensor (Figure 1). Angiosperm suspensors vary widely in size and morphology from a single cell to a massive column of several hundred cells (Meinke 1991; Yeung and Meinke 1993). The suspensor is generally considered to form the attachment between the embryo proper and the donor tissue (Schwartz et al. 1997). In addition, the suspensor provides nutrients and growth regulators for the embryo proper during the early stage of embryogenesis, after which the suspensor degenerates (Schwartz et al. 1997; Beers 1997; Wredle et al. 2001). In most species, degeneration of the suspensor involves programmed cell death (PCD) that is initiated at the base of the suspensor at the late heart-shaped or early torpedo-shaped stages, and eventually consumes the entire suspensor (Raghavan 1986).

Because the embryo of the scarlet runner bean (*Phaseolus coccineus*) is larger than those of *Arabidopsis* and tobacco, it has been selected for studying the interactions between the embryo proper and the suspensor. In the scarlet runner bean, suspensor cells synthesize RNA and protein much more actively than cells of the embryo proper during the early stage of embryogenesis (Walbot et al. 1972; Sussex et al. 1973). Uptake of ^{14}C-labeled sucrose during early embryogenesis occurs initially in the suspensor and thereafter in the embryo proper (Yeung 1980). This observation indicates that the suspensor supplies nutrients to the embryo proper during early embryogenesis. In cultured immature zygotic embryos at the heart-shaped stage, the presence of the suspensor stimulates the growth of the embryo proper and results in a greater number of plantlets than the embryo proper alone (Yeung and Sussex 1979). The effects of the suspensor on the embryo proper are stage-specific and

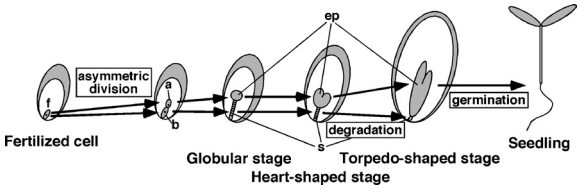

Figure 1. Model scheme of zygotic embryogenesis in angiosperms. a, apical cell; b, basal cell; ep, embryo proper; f, fertilized cell; s, suspensor.

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Abbreviations: CM, conditioned medium; 2,4-D, 2,4-dichlorophenoxyacetic acid; EC, embryogenic cells; GA, gibberellins; 4HBA, 4-hydroxybenzyl alcohol; PCD, programmed cell death; PCV, packed cell volume; 4PMP, 4-[(phenylmethoxy)methyl] phenol; PSK, phytosulfokine; VBE, vanillyl benzyl ether. ‡This article is a part of the memorial lecture by one of the authors (MU), who was awarded the young scientist prize from the Japanese Society for Plant Cell and Molecular Biology in 2004.

are negligible when the embryo proper reaches the torpedo-shaped stage.

In the heart-shaped embryo stage, the activity of gibberellin (GA) in the suspensor is much higher than that in the embryo proper (Alpi et al. 1975). When zygotic embryos from which the suspensors had been surgically removed were treated with GA, development of the embryo proper was enhanced (Yeung and Sussex 1979). GA also plays a role in the embryogenesis of *Tropaeolum* and *Cytisus* (Picciarelli et al. 1984, 1991). In microspore-derived embryos of *Brassica napus*, GA affects the development of the embryo axis (Hays et al. 2002). GA supplied by the suspensor to the embryo proper may be an important factor required for normal embryogenesis.

Two mRNA transcripts, G564 and G541, accumulate specifically within the suspensor of globular-stage embryos of the scarlet runner bean (Weterings et al. 2001). Investigation of the expression and localization of these mRNAs suggests that the apical and basal cells are specified at the molecular level after division of the zygote.

Although the scarlet runner bean is one of the most useful tools to investigate the interaction between the embryo proper and the suspensor, several reports have used other plant species (Schwartz et al. 1997; Ciaviatta et al. 2001; Umehara et al. 2004b). In this review, we summarize recent works on some important factors affecting the development of the embryo proper and the suspensor during early embryogenesis in *Arabidopsis* mutants and during somatic embryogenesis in gymnosperms. In addition, we refer to some of our own researches on somatic embryogenesis in Japanese larch (*Larix leptolepis* Gordon).

Auxin transport between the embryo proper and the suspensor

Auxin is one of the key signaling molecules in the interaction between the embryo proper and the suspensor. It has been proposed to modulate polarization (formation of the apical-basal axis) during early embryogenesis. *Brassica juncea* embryos treated with anti-auxin or an inhibitor of auxin transport during early embryogenesis lose polarity and develop abnormally shaped cotyledons and hypocotyls (Liu et al. 1993; Hadfi et al. 1998). In *Arabidopsis*, the *pin-formed* (*pin*) mutant has been isolated as a mutant of flower phenotype characterized by diminished polar auxin transport (Okada et al. 1991). This phenotype can be mimicked by chemical inhibition of polar auxin transport. The *AtPIN1* gene, which may be expressed as a transmembrane component of the auxin efflux carrier, has been isolated as a causal gene for inducing this phenotype (Gälweiler et al. 1998).

The activity of the synthetic auxin-responsive promoter *DR5* has been used to visualize the spatial pattern of the auxin response, which indirectly reflects the distribution of auxin (Ulmasov et al. 1997; Savatini et al. 1999). By measuring this activity, the role of PIN3 and PIN4 in auxin transport has been characterized (Friml et al. 2002a, 2002b). Recently, Friml et al. (2003) investigated auxin distribution using a reporter gene, *DR5rev::GFP*, to monitor the auxin response and to examine the expression and localization of the *AtPIN* family of genes. During early embryogenesis (at the two-cell stage), PIN1 is located in border membranes between cells of the embryo proper, and auxin is provided to the embryo proper by the suspensor via PIN7. After the 32-cell stage, auxin is synthesized within the apical region of the embryo proper. It is accumulated within the hypophysis via PIN1, PIN3, and PIN4, and is transported to suspensor cells via PIN7. In the early stage of embryogenesis, auxin accumulates in the embryo proper and triggers apical pole specification. In later stage embryos, the direction of auxin transport reverses, and its accumulation within the hypophysis triggers root pole specification.

Genetic analysis of the interactions between the embryo proper and the suspensor using *Arabidopsis thaliana* mutants

In the 1990s, several laboratories used a genetic approach to investigate suspensor development in *Arabidopsis thaliana* by isolating and characterizing recessive mutants exhibiting abnormal suspensor development. The mutants were classified into two categories, (a) *suspensor* (*sus*1, 2) and *raspberry* (*rsy*1, 2), in which the embryo proper possesses an enlarged suspensor and exhibits aberrant development (Schwartz et al. 1994; Yadegari et al. 1994), and (b) *twin* (*twn*1, 2), in which viable secondary embryos arise from suspensor cells (Vernon and Meinke 1994). The presence of these mutants suggests that there is two-way communication between the embryo proper and the suspensor during the early stage of embryo development (Figure 2; Schwartz et al. 1997). In this communication system, the suspensor is considered to support the development of the embryo proper by providing nutrients and growth regulators. Since the suspensor also has a high embryogenic potential, a negative regulatory signal from the embryo proper is required to repress the embryogenic pathway in suspensor cells. Therefore, detailed investigations of the interactions between these two types of tissue are indispensable for elucidating the mechanism of embryogenesis.

Genes that regulate suspensor development have been recently isolated in some mutants of *Arabidopsis*, and

the functions of these genes have been characterized. Specifically, *twn2* has been characterized as follows: the phenotype is caused by a unique regulatory mutation that eliminates the expression of an essential varyl-tRNA synthetase gene within the embryo proper, but not within the suspensor (Zhang and Somerville 1997). Consequently, the embryo proper in the *twn2* mutant degenerates at an early stage, whereas suspensor cells initiate embryonic development and eventually form one or more embryos. Another mutant *twn1* shows the phenotype with defects both in the suspensor and in the cotyledons. Therefore, this causal gene functions not only for maintaining suspensor identity but also for regulating organogenesis in the apex of the embryonic shoot in the late developmental stage of embryo (Vernon et al. 2001).

The *raspberry* mutant *rsy3* shows abnormality in the embryo proper and is arrested in the globular embryo stage of development. *RSY3* encodes a novel protein that is localized in chloroplasts, and it has been suggested that RSY3 protein is required for chloroplast differentiation and embryonic development (Apuya et al. 2002). However, the direct factors produced by the embryo proper to suppress the embryogenic potential of suspensor cells have not been identified in this mutant.

A new model system to investigate the suspensor function during embryogenesis: somatic embryogenesis of conifers

Fertilization and the subsequent development of zygotic embryos occur deep inside both the endosperm and maternal tissue (West and Harada 1993). Because the physical inaccessibility of zygotic embryos makes biochemical and molecular analyses of zygotic embryogenesis difficult, little is known about early events of embryogenesis. However, somatic cells that have embryogenic potential can be used to produce morphologically and developmentally normal mature plants. The process of plantlet induction from somatic cells is called somatic embryogenesis, in which development of somatic embryos closely resembles to that of zygotic embryos. In addition, the spatial and temporal aspects of the programs of gene expression and the accumulation of storage proteins appear to be similar in somatic and zygotic embryos (Borkird et al. 1988; Kiyosue et al. 1992, 1993; Wurtele et al. 1993; Zimmerman 1993).

However, it is difficult to study the interaction between the embryo proper and the suspensor in angiosperms, because somatic embryos of flowering plants do not develop suspensors (Ciaviatta et al. 2001). In contrast to angiosperms, some conifers form well-developed suspensors in *in vitro* embryogenic cultures. Therefore, conifer embryogenic culture should be a useful system for studying suspensor biology.

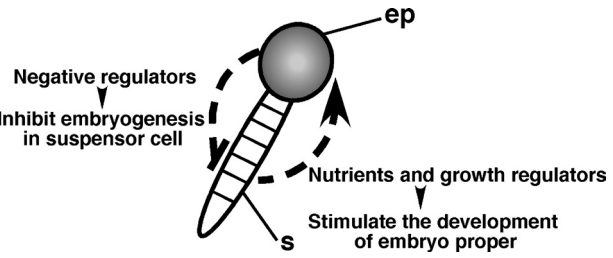

Figure 2. Two-way communication between the embryo proper and the suspensor during early stage of embryogenesis (based on Schwartz et al. 1997). The suspensor provides nutrients and growth regulators to the embryo proper. Conversely, the embryo releases negative regulators that suppress the embryonic potential of suspensor cells, thereby maintaining suspensor cell identity. ep, embryo proper; s, suspensor.

For example, embryogenic culture of gymnosperm was efficiently used to clarify the nature of suspensor-specific gene expression. Ciaviatta et al. (2001) identified a transcript of *PtNIP1;1*, encoding an aquaglyceroporin expressed in early stage zygotic and somatic embryogenesis of loblolly pine (*Pinus taeda* L.). More importantly, this transcript appears to be expressed preferentially within the suspensor. The *PtNIP1;1* promoter has been cloned from loblolly pine, fused to a β-glucuronidase (GUS) reporter gene and used to investigate localization of the expression (Ciaviatta et al. 2002). GUS activity has also been investigated in Norway spruce (*Picea abies* L.) somatic embryos for which a transformation system had been established. This GUS assay has revealed that the *PtNIP1;1* promoter drives early embryogenesis-specific and suspensor-specific gene expression.

The embryogenic culture was also used to clarify the mechanism involved in PCD of the suspensor that occurs in not only in angiosperms but also gymnosperms. Although PCD proceeds only at the distal end of the suspensor in normal somatic embryos of Norway spruce, the actin depolymerization drug latrunculin B prevents normal embryogenesis and results in PCD within the embryo proper (Smertenko et al. 2003). These results suggest that the establishment of actin networks is a vital component of the PCD pathway.

Recently, the biology of the suspensor has been studied using somatic embryos of gymnosperms such as above. It has the possibility that substances regulating the development of the embryo proper or the suspensor (see Figure 2) may be released into culture medium.

Stimulatory factors that modulate somatic embryogenesis

Somatic embryogenesis depends on several modulatory substances, some of which accumulate in culture medium (conditioned medium, CM). From CM of *Asparagus officinalis* L., Matsubayashi and Sakagami

(1996) isolated phytosulfokine-α (PSK), the sulfated pentapeptide H-Tyr(SO_3H)-Ile-Tyr(SO_3H)-Thr-Gln-OH, that 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*, *Zinnia elegans*, and asparagus (Matsubayashi et al. 1997, 1999b; Yang et al. 2001). PSK has been detected in CM of cultures of several plant species using an antibody that specifically recognizes PSK (Matsubayashi et al. 1999a; Hanai et al. 2000; Chen et al. 2000; Eun et al. 2003). Since the general characteristics and effects of PSK have been reviewed elsewhere (Yang et al. 2000; Matsubayashi et al. 2001), only the effect of PSK during embryogenesis is mentioned here.

In carrot, exogenously applied PSK stimulates somatic embryogenesis by activating cell division of embryogenic cells (EC; Kobayahsi et al. 1999a). CM derived from carrot somatic embryo culture markedly stimulates somatic embryo formation, and the active substance within this CM has been purified and identified as PSK (Hanai et al. 2000). The concentration of PSK in this culture was 3.3 nM. In gymnosperms, somatic embryogenesis in *Cryptomeria japonica* is stimulated by PSK (Igasaki et al. 2003), and PSK precursor gene has been identified in *C. japonica* (Igasaki et al. 2003). In an attempt to characterize the role of PSK in gymnosperm embryogenesis, we examined the effects of PSK on somatic embryogenesis of Japanese larch using the culture system established by Ogita et al. (1997, 1999), which is relatively straightforward to induce somatic embryos (comprising the embryo proper and the suspensor), and plantlets can be regenerated readily (Figure 3). In this species, the optimum cell density for induction of somatic embryos is 0.5 ml packed cell volume (PCV) l^{-1}. Substantially fewer somatic embryos form in cultures with a low cell density (0.05 ml PCV l^{-1}; Umehara et al. 2004a). The addition of PSK stimulates somatic embryogenesis, particularly the development of the suspensor region, in low-cell-density culture (Umehara et al. 2005b). Furthermore, the fact that PSK-treated embryos that lack the own suspensor can regenerate the suspensor and continue to develop is further evidence of the stimulatory role of PSK (Umehara et al. 2005b). In contrast, PSK does not stimulate somatic embryogenesis at optimum cell density, 0.5 ml PCV l^{-1}, although it markedly increases the total number of cells in both 0.05 and 0.5 ml PCV l^{-1} culture. These results indicate that inhibitory factors for somatic embryogenesis accumulate in high-cell-density culture. Because the concentration of PSK in CM from suspension culture at the optimum density for embryo formation (measured using a competitive enzyme-linked immunosorbent assay) was very low (less than 1 nM), it is possible that an inhibitory conditioning factor(s) produced by EC may compete with PSK, or may neutralize the stimulatory effects of PSK. Therefore, the effects of such inhibitory factors should be taken into account when using PSK as a growth regulator in tissue cultures. Factors that inhibit somatic embryogenesis in Japanese larch are described in the next section.

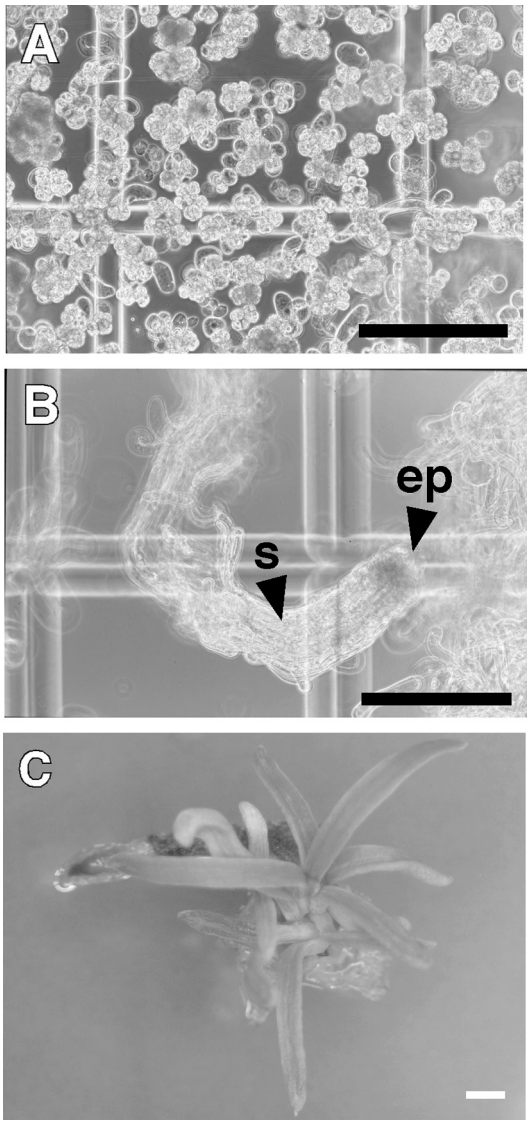

Figure 3. Suspension culture of Japanese larch (*Larix leptolepis* Gordon). (A) Embryogenic cells induced from immature zygotic embryos. (B) A somatic embryo. ep, embryo proper; s, suspensor. (C) A plant regenerated from a somatic embryo. Black bars and white bar indicate 500 μm and 1 mm, respectively.

We suspect that PSK stimulates the vacuolation of cells in the basal region of embryos proper and causes elongation of the suspensors. Because suspensor cells do not exhibit cell division, it is likely that PSK stimulates the differentiation of cytoplasmically dense cells into vacuolated suspensor cells within the embryo proper (Umehara et al. 2005b). PSK generally activates cell division (Mastubayashi and Sakagami 1996;

Matsubayashi et al. 1997; Yang et al. 2001). In carrot, somatic embryogenesis is stimulated by the activation of cell division during embryogenesis when EC is treated with PSK (Kobayashi et al. 1999a). However, in Japanese larch, PSK affects not only cell division but also cell differentiation and cell fate i.e. development of suspensor. Therefore, the effects of PSK on somatic embryogenesis differ in high- and low-cell density cultures.

Furthermore, the presence of auxin and cytokinin is required to produce PSK in *Asparagus*, and each of auxin, cytokinin and PSK is required to start cell division in low-cell-density culture (Matsubayashi et al. 1999a). In carrot, PSK requires auxin to stimulate cell proliferation (Eun et al. 2003). These results indicate that the production and the expression of biological activity of PSK are closely correlated with the signal transduction pathway mediated by auxin and cytokinin. Therefore, it will be necessary to investigate the production and the behavior of PSK in auxin transport between embryo proper and suspensor.

Inhibitory factors that modulate somatic embryogenesis

In carrot, auxin, especially 2,4-dichlorophenoxyacetic acid (2,4-D), is one of the most important factors in somatic embryogenesis (Bellincampi and Morpurgo 1989). When explants are cultured in medium containing 2,4-D, EC are induced and proliferate (Kamada and Harada 1979). After transfer of EC to medium without 2,4-D, somatic embryos form. Therefore, for induction of somatic embryos, EC should be cultured in the absence of 2,4-D. Because cultured cells produce inhibitory factors of somatic embryogenesis endogenously, the efficiency of somatic embryogenesis can be improved by adding activated charcoal, which absorbs inhibitory factors, to the culture medium. In carrot, various phenolic compounds are accumulated in culture medium when charcoal is not present (Fridborg et al. 1978). For this reason, phenolic compounds have long been thought to inhibit somatic embryogenesis in carrot.

In carrot, somatic embryogenesis is strongly inhibited at high-cell-density culture by the release from cells into the medium of inhibitory factors, even in auxin-free medium (Higashi et al. 1998). An inhibitory factor isolated from the medium was firstly characterized as a 4-hydroxybenzyl alcohol (4HBA; Kobayashi et al. 2000a). 4HBA accumulated rapidly in the medium and specifically inhibits rapid cell division during the early globular stage of somatic embryogenesis (Kobayashi et al. 1999b, 2000b, 2001). While 4HBA inhibits embryogenesis in carrot, this factor does not inhibit somatic embryogenesis in several other species including Japanese larch (data not shown). These facts suggest that inhibitory factors may vary among species.

Therefore, it is necessary to use more suitable materials in order to investigate inhibitory factors on the interaction between the embryo proper and the suspensor. In some conifers, somatic embryogenesis is strongly inhibited when cell density is high (Ogita et al. 2000). Using the somatic embryogenesis system of Japanese larch, we studied the role of inhibitory factors. Some conditioning factors suppress somatic embryogenesis in Japanese larch by blocking the development of the suspensor under high-cell-density conditions (Umehara et al. 2004a). No inhibitory factors have been isolated from this species, although several synthetic and natural compounds have been isolated from carrot somatic embryos (Fridborg et al. 1978; LoSciavo et al. 1986; Baldan et al. 1995; Capitano et al. 1997; Kobayashi et al. 2000a). We purified and chemically characterized a novel inhibitory conditioning factor, vanillyl benzyl ether (VBE), from the embryogenic culture medium of this species (Umehara et al. 2005a). VBE was accumulated at high concentrations (greater than 10^{-5} M) in high-cell density cultures. Exogenously applied VBE inhibited somatic embryogenesis, especially suspensor development, but this effect was smaller than that of the addition of original CM from high-cell-density culture. Therefore, VBE is a main inhibitory conditioning factor that regulates suspensor development, but other inhibitory factors remain to be discovered. To search the secondary inhibitory factor, somatic embryos were induced in the medium containing each of the other HPLC fractions with 1×10^{-5} M of VBE. As a result, inhibitory effect of a fraction with VBE was much stronger than that of VBE alone. The secondary inhibitory factor involved in this fraction was chemically characterized as 4-[(phenylmethoxy)methyl] phenol (4PMP), which had a similar chemical structure to VBE (Umehara et al. 2005c). Inhibitory effect of 4PMP with VBE was comparable to the addition of CM from high-cell-density culture. In the future, it is necessary to investigate unknown effective concentration and amounts in the medium of 4PMP, and examine effects of combination with PSK or VBE.

Perspectives

Based on the studies described above, we propose the following hypothesis on the physiological role of PSK, VBE and 4PMP during zygotic embryogenesis in Japanese larch (Figure 4). In gymnosperms, the suspensor has a role to provide nutrients and growth regulators modulating embryonic development as postulated previously for angiosperm embryogenesis (Figure 2). However, apical cells, which are unique to gymnosperms, are considered to promote the

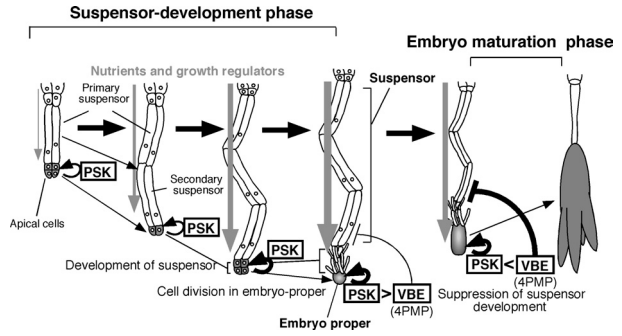

Figure 4. Hypothetical model of the roles of vanillyl benzyl ether (VBE) and phytosulfokine-α (PSK) during zygotic embryogenesis in Japanese larch (*Larix leptolepis* Gordon).

development of both the embryo proper and the suspensor through the production of PSK. While the mitotic activity of the cells that comprise the embryo proper is enhanced by PSK during embryogenesis, production of benzyl ether compounds such as VBE and 4PMP in the embryo proper increases gradually, and they accumulated in the seed during the later stages of embryogenesis suppresses the development of the suspensor and finally causes its degradation. It is unknown whether VBE and 4PMP affects PCD in the suspensor cells as Norway spruce, but the degradation of the well-developed suspensor will provide a space for development and maturation of the embryo proper. Substances such as PSK, VBE and 4PMP may control the length of suspensor and determine the position of the embryo within the seed. However, no information is available on the production of these substances during zygotic embryo formation in Japanese larch. Consequently, it will be necessary to investigate such changes in zygotic embryos to fully understand embryogenesis in gymnosperms.

The function of the suspensor has been recently discussed within the framework of gymnosperm culture systems (Ciaviatta et al. 2001, 2002; Umehara et al. 2005a, 2005b). We attempted to study the biology of the suspensor using the somatic embryogenesis model system of Japanese larch. There are several common physiological traits among angiosperms and gymnosperms. In the future, the availability of gymnosperm culture systems, in which somatic embryos exhibit a well-developed suspensor, should facilitate the elucidation of the general role of the suspensor in embryogenesis of higher plants.

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