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An *in vitro* Culture System used to Investigate Possible Interactions between the Embryo Proper and the Suspensor in Embryogenesis in Japanese Larch (*Larix leptolepis* GORDON)

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Abstract

It has been proposed that the suspensor has important roles in early embryogenesis in seed plants. However, the roles of the suspensor are not well understood, because the development of zygotic embryos normally occurs deep within both the endosperm and the maternal cells. In this paper, we report the development of an *in vitro* culture system to investigate the roles of the suspensor in the development of the embryo proper, using a somatic embryogenesis system with Japanese larch (*Larix leptolepis* GORDON). Our results indicate that the suspensor is essential for the normal development of somatic embryos of this species. This method provides a useful experimental system to investigate the interactions between the embryo proper and the suspensor.

Key words: embryo proper, Japanese larch, somatic embryogenesis, suspensor.

Abbreviations

ABA, abscisic acid; BAP, benzylaminopurine; 2,4-D, 2,4-dichlorophenoxyacetic acid; EC, embryogenic cells; mCD medium, modified Campbell and Durzan's medium; PCV, packed cell volume

In flowering plants, a fertilized cell usually divides transversely and asymmetrically to form a terminal cell, which gives rise to the embryoproper, and a basal cell, which often divides rapidly to form a structure known as the suspensor (Meinke, 1991; Yeung and Meinke, 1993; Willemsen *et al.*, 1998). 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). It is thought that the role of the suspensor is the attachment of the embryo proper to the maternal tissue and the supply of nutrients and growth regulators to the embryo proper from maternal tissues (Sussex *et al.*, 1973; Schwartz *et al.*, 1997).

In *Phaseolus coccineus*, the presence of the suspensor stimulates the growth of the embryo proper, resulting in a greater number of plantlets when embryos are cultured *in vitro* at the heart-shaped stage. The effect of the suspensor on the growth of the embryo proper is stage-specific, weakening when the embryo proper reaches the torpedo-shaped stage (Yeung and Sussex, 1979). In *Arabidopsis thaliana*, several mutants with abnormalities in suspensor development were identified using a genetic approach (Marsden and Meinke, 1985). The mutants identified fall into two classes: (1) *suspensor* (*sus*) and *raspberry* (*rsy*) mutants, which possess an enlarged suspensor and exhibit aberrant development of the embryo proper (Schwartz *et al.*, 1994; Yadegari *et al.*, 1994); and (2) *twin* (*twn*) mutants, from which viable secondary embryos arise from cells of the suspensor (Vernon and Meinke, 1994).

These results suggest that there may be two-way communication between the embryo proper and the suspensor during early embryo development (Schwartz *et al.*, 1997). The suspensor supports development of the embryo proper by providing nutrients and growth regulators (Yeung, 1980; Nagl,

1990). However, the suspensor may have high embryogenic potential, and a negative regulation signal(s) from the embryo proper may be required for maintenance of suspensor cell identity by repression of the embryogenic pathway (Vernon and Meinke, 1994; Schwartz *et al.*, 1997). It is of great importance to investigate the possible interactions between the embryo proper and the suspensor. The molecular nature of the potential signal(s) has not yet been elucidated.

The fertilization and subsequent development of zygotic embryos normally occur deep within both endosperm and maternal cells (West and Harada, 1993). This physical inaccessibility to zygotic embryos causes significant difficulties in biochemical and molecular analyses of zygotic embryogenesis. Therefore, the induction and formation of somatic embryos is a highly useful process for analyzing the steps in zygotic embryogenesis (Zimmerman, 1993).

Although carrot and certain other angiosperms are commonly used in somatic embryogenesis studies, angiosperm somatic embryos do not have suspensors. In contrast, somatic embryos from some conifer species consist of the embryo proper and the suspensor (Ciavatta *et al.*, 2001). Therefore, we attempted to investigate the interaction of the embryo proper and the suspensor using somatic embryogenesis in Japanese larch (*Larix leptolepis* GORDON), for which it is straightforward to induce somatic embryos and regenerate plantlets.

The embryogenic cells (EC) of Japanese larch used in this study were prepared as described previously (Ogita *et al.*, 1997). EC were maintained in three-week subcultures in a modified Campbell and Durzan's basal medium (mCD medium) (Campbell and Durzan, 1975) supplemented with 7 μM 2,4-D, 3 μM BAP, and 3% (w/v) sucrose. The pH of the medium was adjusted to 5.6 before autoclaving at 121℃ for 15 minutes. To induce the development of somatic embryos, EC of 100-250 μm in diameter were collected by passing the culture medium through stainless-steel sieves with pore sizes of 250 and 100 μm. The initial cell density required to induce somatic Japanese larch embryos has already been researched in detail (Umehara *et al.*, 2004). The EC were rinsed five times with phytohormone-free mCD medium, collected by centrifugation at 100 g, and suspended in 10 ml of phytohormone-free mCD medium in 100-ml flasks at cell densities of 0.5 ml packed cell volume (PCV) centrifuged at 100 g per liter of medium. The cells were cultured on a gyratory shaker (100 rpm) at 25℃ under a 16-hour photoperiod provided by fluorescent lamps (white light at 30 μmol photons $m^{-2}s^{-1}$).

Pro-embryos obtained as described above (**Fig. 1A**) were cultured on solidified mCD medium containing 3 g l^{-1} gellan gum, 50 μM ABA, and 3% (w/v) sucrose (maturation medium) for six weeks until maturity (Ogita *et al.*, 1999). The mature embryos (**Fig. 1B**) were cultured on fresh phytohormone-free solidified mCD medium containing 2 g l^{-1} gellan gum and 3% (w/v) sucrose (regeneration medium). After six weeks, the regenerated plantlets were observed at high frequency (**Fig. 1C**, **Table 1**). To investigate the roles of the suspensor in normal embryogenesis, pro-embryos were manually dissected into the embryo proper and the suspensor tissues using a dissecting microscope (**Fig. 1D**), and each dissected tissue was cultured separately on maturation medium. After six weeks of culture, the dissected embryo proper turned brown and ceased development (**Fig. 1G**), whereas the

Table 1 Further development of manually dissected embryo-proper arid suspensor in culture.

Cultured tissues	Frequency of pro-embryos developed (No. of mature embryos/No. of samples)	Frequency of plantlets regenerated (No. of plantlets/No. of samples)
Experiment 1		
*Intact somatic embryos	78% (73/93)	58% (54/93)
Embryo-proper	0% (0/48)	0% (0/48)
Suspensor	0% (0/50)	0% (0/50)
**Embryo-proper + Suspensor	45% (28/62)	39% (24/62)
Experiment 2		
*Intact somatic embryos	80% (87/109)	53% (58/109)
Embryo-proper	8% (5/60)	0% (0/60)
Suspensor	0% (0/68)	0% (0/68)
**Embryo-proper + Suspensor	43% (30/70)	33% (23/70)

*Somatic embryo cultured without dissection in the medium with 50 μM ABA for 90 days.
**Dissected embryo-proper and suspensor were cultured adjacently in the medium with 50 μM ABA for 90 days.

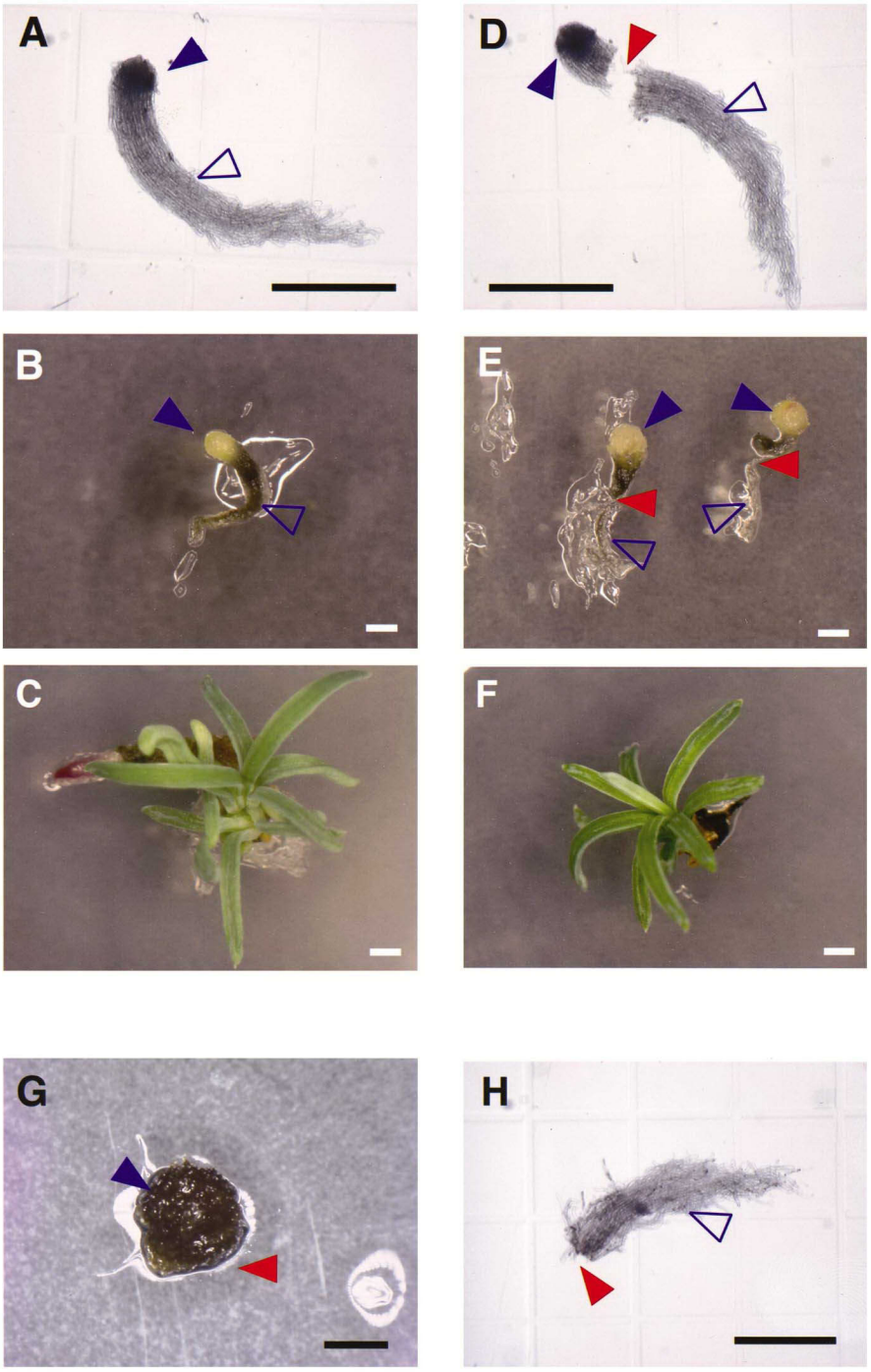

Fig. 1 The essential role(s) of the suspensor in the normal development of somatic embryos of Japanese larch. Pro-embryos that were either left whole or dissected into the embryo proper and suspensor tissues were cultured for six weeks on maturation medium and then six weeks on regeneration medium. Pro-embryos cultured whole (**A**) or after dissection (**D**). Whole (non-dissected) somatic embryos cultured for six weeks on maturation medium (**B**) and then for six weeks on regeneration medium (**C**). A dissected embryo proper and suspensor co-cultured for six weeks on maturation medium (**E**) and then for six weeks on regeneration medium (**F**). A dissected embryo proper (**G**) and suspensor (**H**) cultured for six weeks on maturation medium. Closed blue, open blue, and red arrowheads indicate the embryo proper, the suspensor, and the site of the separation of tissues, respectively. Black and white bars represent 1 mm. These experiments were performed twice with similar results.

embryo-proper of the intact somatic embryos continued normal development (**Fig. 1B**). Even after six weeks, the suspensor looked quite similar to its appearance just after dissection (**Fig. 1D, H**). In contrast, in two independent experiments in which an embryo proper was placed in close proximity to a suspensor on maturation medium, approximately 40% of the embryos proper continued normal development as intact embryos (**Fig. 1E , F; Table 1**).

The importance of the suspensor in embryogenesis has long been discussed (Schwartz *et al.*, 1997). Somatic embryogenesis of Japanese larch is inhibited in cell cultures of high density; the inhibition is caused by the accumulation in the culture medium of factor(s) that suppress suspensor development (Umehara *et al.*, 2004). In this study, most of the embryos proper lacking suspensors did not develop into mature embryos, but when cultured near a dissected suspensor, many developed into mature embryos (**Fig. 1, Table 1**).

These results support the idea that factors that stimulate embryogenesis in gymnosperms are supplied to the embryo proper from the suspensor (Ciavatta *et al.*, 2001). Isolation of the possible factors supporting the development of the embryo proper and characterization of their molecular nature will be crucial in the elucidation of the interactions between the embryo proper and the suspensor. Our method, which utilizes dissection of somatic embryos into the suspensor and embryo proper, will be useful in large-scale biochemistry to identify these possible factors.

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References

Campbell, R.A., Durzan, D.J. 1975. Induction of multiple buds and needles in tissue cultures of *Picea glauca*. Can. J. Bot., **53**: 1652-1657.

Ciavatta, V.T., Morillon, R., Pullman, G.S., Chrispeels, M.J., Cairney, J. 2001. An aquaglyceroporin is abundantly expressed early in the development of the suspensor and the embryo proper of loblolly pine. Plant Physiol., **127**: 1556-1567.

Marsden, M.P.F., Meinke, D.W. 1985. Abnormal development of the suspensor in an embryo-lethal mutant of *Arabidopsis thaliana*. Am. J. Bot., **72**: 1801-1812.

Meinke, D.W. 1991. Perspectives on genetic analysis of plant morphogenesis. Plant Cell, 3: 857-866.

Nagl, W. 1990. Translocation of putrescine in the ovule, suspensor and embryo of *Phaseolus coccineus*. J. Plant Physiol. **136**: 587-591.

Ogita, S., Kubo, T., Fushitani, M. 1997. Anatomical characteristics in early embryogenesis from embryo of *Larix* leptolepis. Forest Res. Environment, **35**: 45-51.

Ogita, S., Sasamoto, H., Kubo, T. 1999. Maturation and plant recovery from embryogenic cells of Japanese larch: effect of abscisic acid in relation to their morphology. J. Forest Res., **4**: 241-244.

Schwartz, B.W., Yeung, E.C., Meinke, D.W. 1994. Distribution of morphogenesis and transformation of the suspensor in abnormal suspensor mutants of *Arabidopsis thaliana*. Development, **120**: 3235-3245.

Schwartz, B.W., Daniel, S., Vernon, M., Meinke, D.W. 1997. Development of the suspensor: differentiation, communication, and programmed cell death during plant embryogenesis. In Cellular and Molecular Biology of Plant Seed Development, Kluwer Academic Publishers, pp. 53-72.

Sussex, I., Clutter, M., Walbot, V., Brady, T. 1973. Biosynthetic activity of the suspensor of *Phaseolus coccineus*. Caryologia **25 suppl**: S262-272.

Umehara, M., Ogita S., Sasamoto, H., Kamada, H. 2004. Inhibitory factor(s) of somatic embryogenesis-regulated suspensor differentiation in suspension culture of Japanese larch (*Larix leptolepis* GORDON). Plant Biotechnol., **21**: 87-94.

Vernon, D.M., Meinke, D.W. 1994. Embryogenic transformation of the suspensor in *twin*, a polyembryonic mutant of *Arabidopsis thaliana*. Dev. Biol., **165**: 566-573.

West, M.A.L., Harada. J.J. 1993. Embryogenesis in higher plants. An overview. Plant Cell, **5**: 1361-1369.

Willemsen, V., Wolkenfelt, H., de Vrieze, G., Weisbeek, P., Scheres, B. 1998. The HOBBIT gene is required for formation of the root meristem in the *Arabidopsis* embryo. Development, **125**: 521-531.

Yadegari, R., de Paiva, G.R., Laux, T., Koltunow, A.M., Apuya, N., Zimmerman, J.L., Fischer, R.L. Harada, J.J., Goldberg, R.B. 1994. Cell differentiation and morphogenesis are uncoupled in *Arabidopsis raspberry* embryos. Plant Cell, **6**: 1713-1729.

Yeung, E.C. 1980. Embryogeny of *Phaseolus*: the role of the suspensor. Z. Pflanzenphysiol. **96**: 17-28.

Yeung, E.C. and Meinke, D.W. 1993. Embryogenesis in angiosperms: Development of the suspensor. Plant Cell **5**: 1371-1381.

Yeung, E.C., Sussex, I.M. 1979. Embryology of *Phaseolus coccineus*: the suspensor and the growth of the embryo proper *in vitro*. Z. Pflanzenphysiol., **91**: 423-433.

Zimmerman, J.L. 1993. Somatic embryogenesis: a model for early development in higher plants. Plant Cell, **5**: 1411-1423.