Cell Development during Somatic Embryogenesis in *Lithospermum erythrorhizon* Observed by Scanning Electron Microscopy

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ABSTRACT---: ***Scanning electron microscopy observation could be used to describe cell development during somatic embryogenesis in Lithospermum erythrorhizon. The purpose of this study is to provide numbers of important morphological information in order to understand the embryogenesis of this plant. Proembryo was composed of small round cells. Proliferating embryogenic cell (PEC) was composed of small round cells in the center, surrounded by slightly elongated cells at the periphery. Globular somatic embryo composed of small round cells. Its surface was covered by extracellular material. Heart somatic embryo was composed of small round cells. Slightly elongated cells in pre-root apex and round cells in pre-shoot apex of early torpedo somatic embryo showing polarization of somatic embryo. In late torpedo somatic embryos, pre-root apex became firm and pre-shoot apex still consisted of round cells.***

Keywords--- *Lithospermum erythrorhizon*, somatic embryogenesis, morphology, scanning electron microscopy

1. INTRODUCTION

The genus *Lithospermum* belongs to Boraginaceae, which are perennial plants. *Lithospermum erythrorhizon* is a representative source of shikonin derivatives (the red naphthoquinon pigments of the root) that have antibacterial, anti-inflammatory and anti-tumor activities. Its root is an important crude drug source in Asian countries (Yazaki et al., 1998). Due to its medicinal importance and its indiscriminate collection from the wild, *L. erythrorhizon* is now rare and endangered plant in Japan (Yazaki et al., 1997).

Recently, plant transformation of L. erythrorhizon to increase the shikonin has been investigated by Yazaki et al. (1998). Nevertheless, the establishement of a reliable procedure for regenerating plants from L. erythrorhizon cell and tissue culture is essential for the recovery of transgenic plants. Therefore, an effort to regenerate this plant using tissue culture method is needed. Yu et al. (1997) carried out plant regeneration from callus culture of L. erythrorhizon. The research investigated the effects of auxin (2,4-D, IAA, picloram and NAA) and cytokinins (BAP and Kinetin) on organogenesis and somatic embryogenesis. However, morphologically, regeneration mostly has been via organogenesis.

In somatic embryogenesis, shoot and root meristems are initiated in one step. Thus, regeneration of plants through somatic embryogenesis is often more advantageous than organogenesis. In some cases, plant regenerated from somatic embryogenesis may develop from a single cell and exhibit less variability (Chee, 1995). These advantages making plant transformation more efficient.

Mariani et al. (1998) used SEM (Scanning electron microscopy) to observe change in surface structure during direct somatic embryogenesis in rice scutellum. Each stage of somatic embryo has its own characteristic on the surface structure. The surface of proembryo was smooth. Cellular microfibril, with its granular structure, was observed on the fibrillar material, which covered the nodular structure surface. Fibrillar material was gradually replaced by mesh-like structure on the surface of a globular stage.

In this study, we observed morphology of proembyo up to torpedo stage of Lithospermum erythrorhizon by scanning electron microscopy. The purpose of this study is to provide numbers of important morphological information in order to understand the embryogenesis of this plant.

2. MATERIAL AND METHOD

Material

Seeds of Lithospermum erythrorhizon provided by Dr. Kazufumi Yazaki (Kyoto University, Japan) were used as the material of this experiment.

Methods

1. Shoot culture in vitro

The seeds were surface sterilized in 1% Na-hupochlorite for 20 min. The seeds were then rinsed 3 times with sterilized, distilled water and germinated on sterilized, moistened cotton. The cotyledon part was dissected from the germinated seedling and sown on the shoot multiplication medium (SMM). The SMM consisting of Murashige and Skoog (MS) basal medium supplemented with 0.5 µM NAA, 5 µM BAP, 3% sucrose and solidified with 0.8% agar. The pH of the medium was adjusted to 5.8 prior to autoclaving for 15 min at 121^{o}C. After the shoots developed, they were subcultured on the SMM every months for shoot multiplication. The cultures were kept under continuous light at 25^{o}C.

2. Calli induction

Petioles of shoot cultures in vitro were used as the explants for calli induction. The petioles were cut into several segments, then they were inculated on calli induction medium (CIM 1 and CIM2). The CIM 1 consisiting of MS Basal medium supplemented with 10 µM NAA, 10 µM Kinetin and 1.5 g/l proline, 3% sucrose and solidified with 0.8% agar. The CIM 2 consisting of M9 Basal medium supplemented with 10 µM NAA, 10 µM Kinetin and 1.5 g/l proline, 3% sucrose and solidified with 0.8% agar. The cultures were kept in darkness at 25^{o}C. The yellowish friable calli were used as source of inocula for embryogenic cell culture.

3. Somatic embryo induction, development and germination

For the initiation of embryogenic cell culture and somatic embryo induction, the yellowish friable calli in CIM 1 were inoculated in embryogenic cell culture medium (L2) for 1 week and subsequently subcultured in embryo induction medium (L3) for 3 weeks. Thereafter, the proembryos, globular and heart somatic embryos were subcultured in embryo development medium (L4) for 3 weeks. Subsequently, the developed somatic embryos were subcultured in embryo germination medium (L5). The composition of media is shown in table 1. All cutures were conducted in 125 ml flasks containing 25 ml media on rotary shaker at 100 rpm. All the cultures were maintained in the darkness at 25^{o}C.

Table 1. Somatic embryogenesis medium using MS Basal medium

Medium	Plant Growth Regulator		Supplement	Carbon Source
	NAA	Kinetin		
L2	5 µM	0.5 µM	1.5 g/l proline	3% sucrose
L3	5 µM	5 µM	1.5 g/l proline	3% sucrose
L4	1 µM	1 µM	1.5 g/l proline	3% sucrose

4. Scanning electron microscopy (SEM)

The morphogenic developments of somatic embryos were observed by SEM. The samples were fixed with 5% glutaraldehyde in cacodylate buffer pH 7.2 at 4 ^{o}C for 24 hr. The samples then were rinsed in 0.1 M cacodylate buffer 3 times. Thereafter, the samples gradually dehydrated in an ascending alcohol series; immersed in isoamyl acetate for 5 min and dried at critical point drying apparatus DCP-1 (Denton Vacuum), using CO2 as the transient fluid. Finally, the samples were coated with gold by an ion sputtering apparatus Jeol JEE and observed by SEM Philips XL 20

3. RESULT AND DISCUSSION

1. Shoot cultures in vitro

Seeds of Lithospermum erythrorhizon could germinate after 5 to 7 days sown on sterilized, moistened cotton (Fig. 1a). Then, the cotyledon part was dissected from the germinated seedling and sown on the shoot multiplication medium (SMM). After 2 weeks of culture on SMM, the shoots developed (Fig. 1b). The shoots multiplicated during continuous subculture on SMM and were used as the explants source for calli induction (Fig. 1c).

2. Calli induction

Calli induction was conducted on CIM1 and CIM2 solid media. Friable calli were formed (Figs 2a and 2b) after 3 weeks of culture and used as inoculums for embryogenic cell cultures.

Fig.1. Germinated seed of *Lithospermum erythrorhizon*, Shoots developed after 2 weeks of culture on SMM, shoot culture *in vitro*

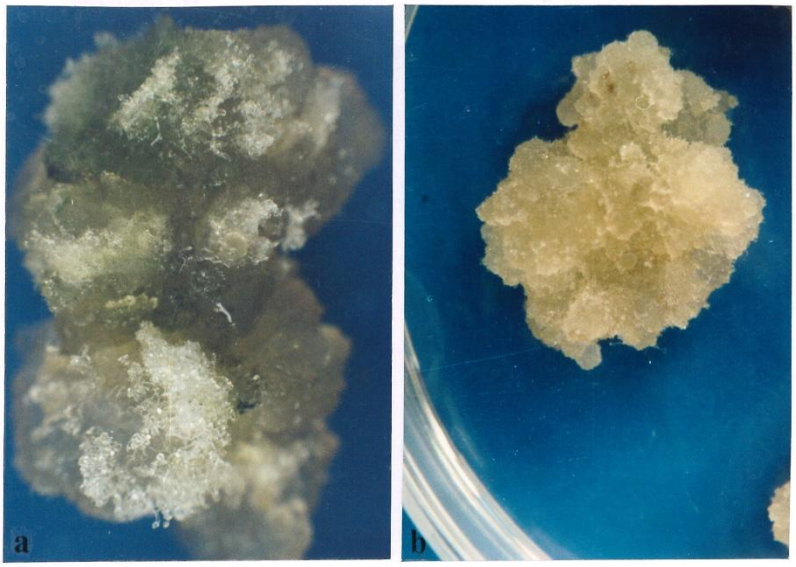

Fig. 2. Friable calli were formed on CIM1 (a) and CIM2 (b) solid media after 3 weeks of culture

3. Somatic embryogenesis

The result of somatic embryogenesis is shown in table 2.

Inoculum	Medium	Stages of somatic embryo	Time
Callus on CIM1	L2	Proembryo, Proliferating embryogenic cell (PEC)	1 week
	L3	Globular, heart	3 weeks
	L4	Early and late torpedo	3 weeks

In L2 medium, the single cells derived from callus on CIM1 developed into proembryos and proliferating embryogenic cells (PEC) within 1 week. By subcuturing the proembryos and PEC into L3 medium, they developed into globular and heart somatic embryos within 3 weeks of culture. Further development of somatic embryos into torpedo stage was achieved when the somatic embryos from l3 medium were subcultured into L4 medium for 3 weeks.

4. SEM observation

Scanning electron micrographs of somatic embryo development in Lithospermum erythrorhizon were shown in Figs. 3A-D and Figs. 4A-D. Single cells derived from callus on CIM1 (Fig. 3A) developed into proembryo that was composed of small round cells (Fig. 3B). Mariani et al. (1998) also reported that proembryo of rice somatic embryo was composed of small round cells.

Proliferating embryogenic cell (PEC) was composed of small round cells in the center, surrounded by slightly elongated cells at the periphery (Fig. 3C and 3D). PEC of embryogenic cultures in Japanese cedar (Maruyama et al., 2000) has the same morphology as that of Lithospermum erythrorhizon.

Globular somatic embryo composed of small round cells (Fig. 4A). Its surface was covered by extracellular material (Fig. 5). Heart somatic embryo was composed of small round cells (Fig. 4B). Cook and Brown (1995) reported that globular and heart somatic embryo of Okra were composed of small round cells. According to Samaj et al. (1995), the chemical composition and structural arrangement of the extracellular material at the cell surface may play fundamental roles in cell recognition, cell to cell interaction, cell division and differentiation.

Slightly elongated cells in pre-root apex and round cells in pre-shoot apex of early torpedo somatic embryo showing polarization of somatic embryo (Fig. 4C). Late torpedo stage developed after 3 weeks of culture. In late torpedo somatic embryos, pre-root apex became firm and pre-shoot apex still consisted of round cells. (Fig. 4D). From this study, it can be concluded that SEM observation could reveal the process and cellular development during somatic embryogenesis. The process underwent sequence as follow: embryogenic cell, proembryo, proliferating embryogenic cell (PEC), globular, heart, torpedo. The shape of cells become mark of cell division and differentiation (cellular development).

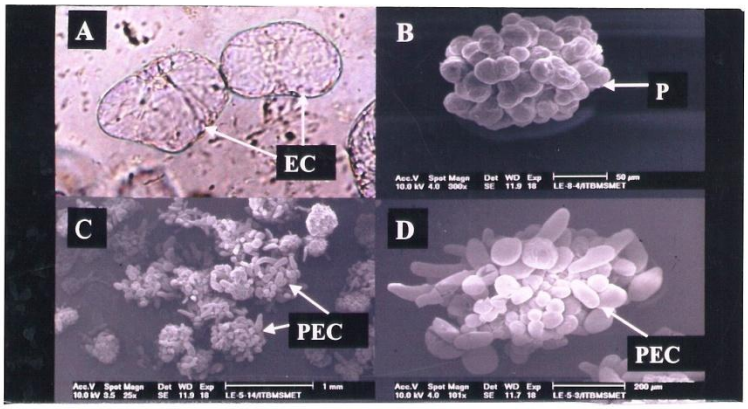

Fig.3 Single embryogenic cell (EC) of *L. erythrorhizon* (A), Proembryo (P) was composed of small round cells (B), Proliferating embryogenic cell (PEC) was composed of small round cells in the center, surrounded by slightly elongated cells at the periphery (C,D).

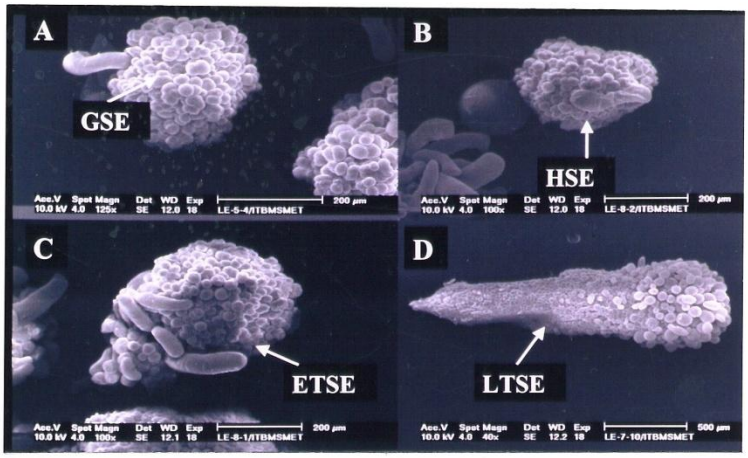

Fig.4 Globular somatic embryo (GSE) composed of small round cells (A), Heart somatic embryo (HSE) was composed of small round cells (B), Slightly elongated cells in pre-root apex and round cells in pre-shoot apex of early torpedo somatic embryo (ETSE) showing polarization of somatic embryo (C), Late torpedo somatic embryo (LTSE) developed 3 weeks of culture on L4 medium (D)

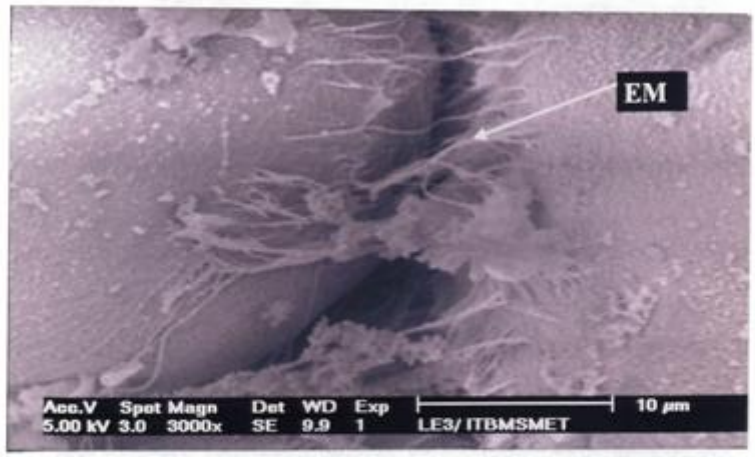

Fig. 5 Globular somatic embryo surface was covered by extracellular material (EM)

4. ACKNOWLEDGEMENT

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