Shoot organogenesis from mulberry leaf tissue: origin and development of regenerated shoots

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Direct shoot organogenesis induced from mulberry leaf tissue was observed histologically. In the initial stage of culture, small meristematic nodules were newly formed from subepidermal layer in the midrib and its adjacent zone of cultured leaf tissues, resulting in dome-like protrusions initiated on the adaxial leaf surface. These protrusions were elongated, from which leaf primordia were differentiated thereafter. In later stage, the cone-shaped cells were also differentiated in the outer surface of leaf primordia, and trichomes with a spheroidal head as well. In regenerated shoots, calcium deposition was observed in idioblasts that changed into rounded form. All of these histological features revealed the direct shoot organogenesis without taking a pathway through callus phase.

Key words: mulberry tissue culture, direct shoot organogenesis, histology

INTRODUCTION

Efficient shoot organogenesis is induced from mulberry leaves in the specified conditions, which is accomplished by a two-step procedure consisting of seedling culture for obtaining the explant source and leaf culture for inducing shoot differentiation (SUGIMURA et al., 1998a, 1999a). The addition of cytokinin and anti-auxin to the medium was essential in each culture step for a high frequency of shoot organogenesis. From shoots formed by such a manner, complete plantlets were regenerated in vitro and grown in pots under outdoor conditions (ADACHI et al., 1999). In this culture system established, it is interesting to examine the process of regenerable shoot differentiation.

In this paper, we describe histological observations on the precise origin and development of shoot buds formed from explanted mulberry leaves based on sectioning of cultured tissues. Scanning electron microscopic (SEM) observations of organogenic process are also reported.

MATERIALS AND METHODS

Plant materials

Mature seeds of mulberry (Morus alba L. cv. Ichinose) were sterilized with 1% sodium hypochloride solution for 20 min, rinsed well with sterile water, and germinated on the solid LS medium (LINSMAIER and SKOOG, 1965) supplemented with 10^-5 M 6-benzylaminopurine (BA) and 10^-7 M 2, 3, 5-triiodobenzoic acid (TIBA). True leaves from the seedlings were used as a source of explants (SUGIMURA et al., 1998a, 1999a). The explants were cultured on the medium supplemented with 10^-5 M thidiazuron (TDZ) and 10^-7 M TIBA. All cultures were maintained under 12 hr photoperiod at 28°C. For light microscopy and SEM observations, explanted...
leaf tissues were sampled at different culture periods.

**Specimen preparation for light microscopy**

Sample tissues were fixed with 3% paraformaldehyde solution overnight. After dehydration by a graded series of ethanol, they were embedded in LR White resin (London Resin Co. Ltd.) and polymerized for 24 hr at 60°C. Sectioning was made by glass knives. All the sections were stained with 0.05% toluidine blue. For the observation of regenerated shoots, harvested fresh tissues were sliced with a hand microtome (NK system, MTH-1), and stained with a silver nitrate solution to detect calcium deposition in idioblasts (SUGIMURA et al., 1998b, 1999b).

**Specimen preparation for scanning electron microscopy**

The basal leaf tissues were fixed with 2.7% glutaraldehyde in 0.1 M phosphate buffer (pH 7.0) overnight and dehydrated in a graded series of acetone at 30 min intervals. After critical point drying, they were sputtered with gold and observed with a scanning electron microscope (Hitachi S-530) at acceleration voltage of 35 KV.

**RESULTS**

Shoot organogenesis was consistently induced from the basal portion of explanted leaf, especially midrib and its neighbouring zone, under the present culture conditions (SUGIMURA et al., 1998a, 1999b). Therefore, transverse sections of leaf tissues including midrib were observed by light microscopy, and adaxial surfaces of the basal tissues were also carefully observed by SEM. Based on both microscopic features, organogenic process was categorized as three stages: (1) Beginning stage, (2) Developmental stage, and (3) Later stage.

**Beginning stage**

As a control, leaves excised from the seedlings grown on the medium with and without growth regulators were observed before culturing. The epidermal cell layer was smoothly expanded in the leaf surface (Fig. 1A). The cone-shaped idioblasts and rounded idioblasts

![Image 1A](image1A.png)

**Fig. 1. Observations of leaf tissue excised from a seedling grown on the medium without growth regulators.** A: SEM of adaxial surface of midrib and its adjacent zone. Bar=100μm. B: Transverse section showing the organization of epidermal, subepidermal and parenchyma cells just above midvein. Bar=80μm. Abbreviations for Fig. 1 to 5: C, callus mass; E, epidermal cell; sE, subepidermal cell; Ib, idioblast; Lp, leaf primordia; Md, meristemoidal dome; Mn, meristematic nodule; Mr, midrib; T, trichome; Vb, vascular bundle.
with a hooked tip were present in the layer, and trichomes with a stalk as well. The presence of idioblasts and trichomes has been reported by Sugimura et al. (1998b, 1999b) and has been attributed as a morphological feature in mulberry leaves. The epidermal cell layer in the sections was confirmed to be smooth, consisting of rectangle cells (Fig.1B). The subepidermal cells were appressed to one another and to the epidermal cells. The vascular bundles of midvein were embedded in the parenchyma.

When the leaves of seedlings grown on BA- and TIBA-containing medium were subcultured on the medium supplemented with TDZ and TIBA, undulating epidermal layer was observed along the adjacent zone of midrib (Fig. 2A). A cluster of slight protrusions was observed on the adaxial side of cultured leaf tissue. Newly-formed meristematic nodules could be found just

Fig. 2. Observations of leaf tissue cultured on the medium supplemented with TDZ and BA. A: SEM of adaxial surface showing epidermal cell layer that was undulated along midrib. Bar=100μm. B: Transverse section showing meristematic nodule newly formed in subepidermal layer. Bar=100 μm. Inset: Magnified meristematic nodule. Bar=40μm.

below epidermal cell layer (Fig. 2B), indicating that meristematic activity was taken place in subepidermal cells. As shown in Fig. 3A, meristematic nodules were enlarged into dome-like protrusions. The sections of dome-like protrusions showed that meristematic cells were actively produced in subepidermal layers (Fig. 3B and 3C). Numerous protrusions were often formed in midrib and its adjacent zone of the basal leaf portion (Fig. 3D). The pre-existing idioblasts and trichomes in explanted leaf tissue were observed on the outer surface of the protrusions, thereby providing evidence that these protrusions were not originated from callus cells. The direct initiation of meristematic protrusions was the first sign of shoot organogenesis. These histological changes were induced within a few days after the start of leaf culture.

**Developmental stage**

The meristematic protrusions were further developed into an elongated form in which leaf primordia were differentiated (Fig. 4A). Each leaf primordium was opened by splitting at the distal end of the elongated form (Fig. 4B and 4C). The leaf primordium was smooth in the outer layer due to the absence of idioblasts and trichomes. Non-organogenic callus masses were observed around the elongated protrusions. The formation of leaf primordium was induced during 4-7 days after culture.

**Later stage**

The sharp, cone-shaped cells were proliferated on the abaxial surface of leaf primordia, and trichomes with a spheroidal head as well (Fig. 4D). Differentiation of these two types of cells is appeared to be closely associated with growth of leaf primordium.

The organization of leaf primordium was confirmed in the sections (Fig. 5), showing that the meristemoidal dome was formed on the sur-

![Fig. 4. SEM of developing meristematic protrusions. A: Elongated protrusion with leaf primordium. Bar=100 μm. B and C: Leaf primordium just opened from the distal end of elongated protrusion. Bars=100 μm. D: Differentiation of cone-shaped cells and trichomes from the abaxial surface of leaf primordium. Bar=100 μm.](image-url)
face of explanted leaves. Multiple shootlets were often regenerated from the dense area of meristematic protrusions (Fig. 6). In regenerated shoots, three types of idioblasts were observed (Fig. 7): (1) cone-shaped idioblasts without Ca deposition, (2) bulbous idioblasts with partial deposition of Ca, and (3) rounded idioblasts with a hooked tip in which full deposition of Ca. Morphological changes and Ca deposition in idioblasts underwent according to shoot development.

**DISCUSSION**

Tissue culture studies in a few plant species have provided information on precise location of cells that undergo direct shoot differentiation de novo. In *Brassica juncea* (SHARMA and BHOJWANI, 1990) and *B. campestris* (HACHEY et al., 1991) cotyledons, shoots were observed to develop from meristematic nodules resulting from the meristematic activity of vascular parenchyma cells. REYNOLDS (1989) reported that adventive organogenesis from stem segments of *Solanum carolinense* arose from miotic activity of the external phloem. Whereas the vascular cells were involved in direct shoot organogenesis in these species, in *Begonia rex* the epidermal cells at the base of glandular hairs developed into bud meristems (CHLYAH and TRAN THANH VAN, 1984). In young cotyledons of *Picea abies* the hypodermal cells as well as the mesophyll cells below them were recognized to give rise to meristems and finally to adventitious buds (BORNMAN, 1983). In mungbean, the epidermal cells at the basal adaxial side of the cotyledonary petiole and the subepidermal cell layer below these competent cells were involved in meristematic nodule formation from which shoots regenerated (MENDOZA et al., 1993). When leaf and cotyledon tissues were used as a source of explants, the origin of direct shoot regeneration possibly could be due to species variation or differences in cultural conditions.

Our reports demonstrated that efficient shoot initiation was induced in only mulberry...
leaves which cultured on the medium supplemented with cytokinin and anti-auxin (SUGIMURA et al., 1998a, 1999b). In this culture conditions, the meristematic nodules were newly formed in the subepidermal layer and developed into shoot regeneration, indicating that the origin of regenerated shoot is meristematic nodules formed in subepidermal layer, not the callus masses. Similar histological features have been reported by OKA and OHYAMA (1981). In tissue culture of mulberry leaf, the competent cells for organogenesis were present in the subepidermal layer situated above midrib and its adjacent tissues, not existing in vascular and mesophyll tissues.

We reported the inducible shoot organogenesis by application of appropriate concentrations of TDZ and TIBA (SUGIMURA et al., 1998a, 1999b). This response may be induced by physiological alterations through (1) the counteraction of the endogenous auxin by the exogenous cytokinin and (2) the blockage of the endogenous auxin influx from the neighbouring tissues by TIBA. No meristematic nodules was observed in leaf explants cultured on the medium excluding TDZ and TIBA. This indicates that these growth regulators are involved in the various organogenic stages from the formation of meristematic protrusions to shoot differentiation and development. Distinct evidence on the location of organogenic cells in mulberry plants and the regeneration site predictable may provide useful information for generating mulberry transformants by Agrobacterium infection and direct gene delivery. In other words, targeting cells for gene
delivery could be specified based on these histological observations.

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REFERENCES

宮崎順司・杉村順夫・小谷英治・古澤泰治：クワ葉組織からの不定芽分化—その起源と発達過程

クワ葉組織からの不定芽形成過程を組織学的に調べた。培養初期段階において、葉底部の中肋及びその周辺組織の副表皮層に小さな分裂組織塊が新生成された。この形成により、上面表皮にドーム状の突出構造が出現した。この突出構造は培養過程に伴い肥大し成長し、葉原基が分化した。培養後期になると、発達した葉原基の表面で角状細胞と球状細胞を含む毛茸組織が分化した。再生したシートには、カルシウムを蓄積した球状の細胞が観察された。これにより、該組織の特徴を、不定芽はカルシウムを経由して形成されるのではない、直接的な器官分化によって形成されることが明らかになった。