Histological and cytological study on meristematic nodule induction and shoot organogenesis in *Paeonia ostii* ‘Feng Dan’

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Abstract

This is the first report concerning the sequence of histological and cytological events occurring during organogenesis from cotyledon-derived meristematic nodules (MNs) in *Paeonia ostii* ‘Feng Dan’. Sections were made and studies were carried out with dissecting microscope, light microscope, scanning electron microscopy (SEM) and transmission electron microscopy observation. Histological studies revealed a complex developmental process of morphogenesis including five stages: (1) callus originated from cell division in both cambial and cortical regions; type I—yellow compact callus with densely arranged clumps was identified as embryogenic callus. (2) pre-nodular structure consisted of organization center (a central area of vascularization surrounded by meristematic cell layers) and an epidermis-like layer; (3) independent MNs comprised of organization center, a cortical-like area of parenchymatous cells and an epidermal-like area; (4) nodular clusters displayed vigorously internal meristematic cell division and generated a relative movement towards the nodules periphery, establishing vascular connection with primordia; (5) successive new elongated shoots with axillary bud primordia were developed. SEM observations showed three types of extracellular matrix, a smooth membranous layer, fibrillar structures and granular mucilage-like secretions on embryogenic callus, demonstrating its dynamic morphological changes. Ultrastructural analysis revealed striking changes of chloroplast morphology and starch content during MNs morphogenesis. This study allows a better understanding of in vitro regeneration via MN culture and provides references for protocol optimization and genetic transformation.

Key message

This report firstly revealed a developmental sequence, dynamic changes of extracellular matrix and ultrastructural characteristics during meristematic nodules morphogenesis in *Paeonia ostii* ‘Feng Dan’ with morpho-histological and cytological analyses.

Keywords *Paeonia ostii* ‘Feng Dan’ · Meristematic nodule · Organogenesis · Histology · Ultrastructure

Abbreviations

| Abbreviation | Description |
|--------------|-------------|
| BA           | 6-Benzyladenine |
| CIM          | Callus induction medium |

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Introduction

Tree peony (Paeonia sect. Moutan) is well-known in China because of its medical, ornamental and oil value (Yu et al. 2016). P. ostii is main option for oil tree peony because of the high yield and adaptability. However, the low efficiency and long cycle of conventional propagation methods, such as grafting and division, severely constrain its breeding and are insufficient to address the increasing commercial demands. Tissue culture is an attractive option to overcome this problem. Recently, an efficient and reproducible in vitro regeneration system via Meristematic nodules (MNs) culture (manuscript PCTO-D-21-00472) have been developed, which can be a feasible system for overcoming various obstacles in the regeneration system of tree peonies published previously, such as vitrification, low multiplication, poor rooting and difficult acclimatization in micropropagation (Beruto et al. 2004; Wen et al. 2020), rare differentiation in callus culture (Zhu et al. 2018), and high deformity rates and low germination in somatic embryogenesis (Du et al. 2020).

MNs comprising of organization centers (OCs), a cortical-like area of parenchymatous cells and an epidermal-like area, have high regeneration potential, and genetic stability, making them attractive for plant regeneration via organogenesis, in vitro phytochemical production and plant transformation (McCown et al. 1988; Batista et al. 2008). Successful in vitro regeneration through MN culture has been reported in several woody and herbaceous plants, such as Eucalyptus globulus (Dobrowolska et al. 2017), Liquidambar orientalis (Bayraktar et al. 2015), Populus euphratica (Ferreira et al. 2009), Humulus lupulus (Fortes and Pais 2000), Sclerocarya birrea (Moyo et al. 2009) and Billbergia zebrina (Dal Vesco et al. 2011).

Histological and cytological analyses of the MN culture system have been carried out on several plants (Aitken-Christie et al. 1988; Batista et al. 2000; Ferreira et al. 2009; Dal Vesco and Guerra 2010), while the origin of MN and their developmental pattern are highly variable according to the species and explant type. Meanwhile, histological research is necessary to provide detail evidence for distinguishing MN from somatic embryo because they superficially resemble each other (Haensch 2004). There were scarce reports of MNs in Paeonia before. Zhong (2011) established a nodule induction and multiplication system of P. rockii through calli induced from petiole sections. Subsequently, Qin et al. (2012a, b) further optimized the system in P. lemoinei ‘Golden Era’ and P. itoh ‘Barzella’ but failed to regenerate complete shoots. Organogenesis from nodules is the major limiting step of the morphogenesis process in their study, thus little information on the correlation of developmental stages with histological and cytological events are available. Therefore, based on the regeneration system we obtained via MN culture (manuscript PCTO-D-21-00472), this study aims to reveal the histological and cytological pattern addressing developmental sequence for induction and differentiation process of MN in P. ostii ‘Feng Dan’, providing critical references for optimization of this system and application of gene transfer.

Materials and methods

Tissue culture

The protocol of meristematic nodule induction and shoot organogenesis used in this study was established under optimal conditions obtained previously (manuscript PCTO-D-21-00472). Mature seeds of P. ostii ‘Feng Dan’ at 90 days after anthesis (DAA) were collected in August 2018 from living adult plants grown in Beijing Guose Peony Garden in Beijing, China (40° 45′ N, 115° 97′ E), and stored in a freezer (−4 °C) for 2 months before use to break dormancy. The sterilization of seeds was same as described in the protocol. The basal media was modified 3g medium (mMS, half-strength macromelakets and full-strength Ca2+) (Murashige and Skoog 1962) and modified woody plant medium (mWPM, double strength of Ca2+) (Lloyd and McCown 1980). All media were supplemented with 3% sucrose and 0.7% agar, and the pH was adjusted to 5.8–6.0 before autoclaving (at 118 kPa and 121 °C for 20 min). All reagents were supplied by Biodee (Beijing, China). The cultures, if not otherwise stated, were maintained at 24 ± 1 °C under a 16 h photoperiod of 50 µmol·m−2·s−1 illumination intensity provided by LED light (70% red light + 30% blue light) (TLD 36 W Philips, Beijing, China).

For callus induction, zygotic embryos were aseptically isolated from seeds and inoculated on germination medium [mMS + 2.57 µM 6-benzyladenine (BA) + 2.89 µM gibberellin (GA3)] for 15 days. Their expanded cotyledons were...
cut into 1 × 1 cm pieces and inoculated on callus induction medium (CIM) [mMS + 4.04 µM N-(2-chloro-4-pyridyl)-N-phenylurea (CPPU) + 5.37 µM α-naphthylacetic acid (NAA)] with the abaxial side touching the medium, under dark conditions. Cultured cotyledons were collected on 0, 5, 10, 15, 20 and 25th day respectively from CIM for morpho-histological examine and histological analysis. Embryogenic callus (EC) and non-embryonic callus (NEC) cultured in CIM for 30 days were collected and distinguished with morpho-histologic and ultrastructural analyses.

For MN induction and shoots differentiation, ECs induced on CIM after 30 days of culture were inoculated into MN/shoot induction medium (MSIM) [mWPM + 2.02 µM CPPU + 2.27 µM thidiazuron (TDZ)] under light condition. Nodules with leaf clusters were developed from ECs on MSIM after 12 subcultures with subculture time of 10 days, and the materials were collected every time before subculture for histological and ultrastructural analyses. Then, these nodules were transferred onto mWPM containing 1.29 µM BA and 0.58 µM GA3 for shoot elongation with subculture time of 30 days.

### Histological analysis

Five days after cultured on CIM, there was no obvious difference in morpho-histological observation of the explants. The cotyledons turned yellow and became swollen (Fig. 1A) due to vigorously division of cortex parenchyma cells (Fig. 1B) after 10 days of culture. Meanwhile, meristematic cell mass with rapid cell division was observed locally around the area of the vascular bundle (Fig. 1C). After 15 days of culture, visible calli formed on the edge of the cotyledons (Fig. 1D). Sectional observation revealed that callus formation was unevenly along the explant tissue (Fig. 1E). The original tissue structure and morphology of some parts of the explant disappeared, and a small proportion of calli expanded into clumps after 20 days (Fig. 1F). The proportion of clumpy calli increased, and gradually covered the entire explant after 25 days. There were two morphologically distinct types of calli after 30 days of induction: type I-yellow compact calli with densely arranged clumps (Fig. 2A) and type II-transparent watery loose calli (Fig. 2B).

### Identification of embryogenic callus (EC) and non-embryonic callus (NEC)

It was observed through SEM that surface cells of type I callus showed a globular shape and tightly packed structure (Fig. 2C), while type II callus exhibited disorganized, elongated and tubular cells (Fig. 2D). Cells of type I callus were small, isodiametric and densely arranged with large and clear nuclei under histological analysis (Fig. 2E), while type II callus were shown to be large, irregular, and highly vacuolated cells with abundance of intercellular space (Fig. 2F). Sectional observation through TEM revealed that cells of type I callus had large nucleus containing prominent nucleoli and chromatin that showed even distribution and little or no visible nucleoli (Fig. 2F). Type I calli tended to form a loose callus tissue (Fig. 2B), while type II calli tended to form a compact callus tissue (Fig. 2A).

### Transmission electron microscopy (TEM)

Fresh samples (callus, MNs, nodular clusters and nodules with differentiated leaf; 2 × 2 × 2 mm) were prefixed in 4% buffered glutaraldehyde (0.1 M phosphate buffer, pH 7.2) for 2 h at room temperature. The samples were rinsed in the same buffer with four changes (15 min each) and post fixed in buffered 1% OsO4 at 4 °C overnight. After rinsing in distilled water, the samples were dehydrated in a graded acetone series (50, 70, 80, 90, and 100%) with 20 min per level and embedded in Epoxy resin. Ultrathin sections were cut to 70 nm on a LKB-5 ultramicrotome, stained with uranyl acetate and lead citrate, and examined with a TEM (JEM-1200 EX).
no condensation, a few small scattered vacuoles, abundant mitochondria, and chloroplasts were degraded to plastids with starch grains accumulation in which the endometrial system was disassembled (Fig. 3A). In contrast, cells of type II callus showed a large vacuole occupying nearly the whole cytoplasmic space, the nucleus and other cytoplasmic organelles located in a narrow strip of cytoplasm between the cell wall and the large vacuole (Fig. 3B). Besides, no starch grain was observed. Type I callus cultured in CIM obtained regenerated shoots ultimately, while Type II callus was gradually browned without differentiation. In summary, type I callus was identified as EC.

**MN induction and organogenesis**

Histological and ultrastructural study revealed a developmental sequence leading to the formation of MNs and shoot differentiation presented through the following stages:

1. **Pre-nodular structures:** Cells on the surface of the callus were organized into small units under SEM observation after 1–2 subcultures in MSIM (Fig. 4A). Correspondingly, the external layer of the callus was composed of small, isodiametric, densely stained meristematic cells which were organized into abundant meristematic cell masses in peripheral regions (Fig. 4B). During 3–4 subcultures, there were visible small protuberances observed on callus, which were termed as pre-nodular structures (Fig. 4C). Under histological analysis, neo-formed tracheary elements developed inside of meristematic cell mass and organization centers (OCs) consisting of a central area of vascularization surrounded by meristematic cell layers with vigorous division were observed (Fig. 4D). Later, the OCs became autonomous and developed an epidermis-like layer.

2. **MNs:** After 5–6 subcultures, the pre-nodular structures greatly increased in diameter due to vigorous divisions in meristematic cells surrounding vascularized centers, and rapidly formed conspicuous large protuberances (Fig. 4E) differentiating into a more defined internal structure. Histological sections revealed the large protuberance comprising of OCs, a cortical-like area of parenchymatous cells and an epidermal-like area (Fig. 4F), and there were two types of OCs, linear and nested (Fig. 4G, H). These typical features account for their classification as MNs. Smaller nucleus and larger vacuoles in cells of MNs were observed under TEM observation, and less starch grains exhibited in the plastids, where containing moderately developed lamellar structures (Fig. 4I, J).

3. **Nodular clusters:** During 7–8 subcultures, enlargement of nodules in size were accompanied by the formation of indentations created by differential expansion of multiple OCs, that appeared initially as small grooves on the surface of the nodules, and then progressively deepened, yet nodule break-up was never observed. In
the same way, smaller ‘daughter nodules’ were produced without detachment. Thus, several MNs displaying different levels of development were loosely attached to each other and developed into nodular clusters in appearance (Fig. 5A). Under histological analysis, meristematic cells of OCs inside the nodules intensely divided and showed relative movement towards the nodules periphery (Fig. 5B). Smaller
nucleus and larger vacuoles were also observed in cells of nodular clusters under TEM, nevertheless, plastids contained moderately developed lamellar structures without starch grain (Fig. 5C). During 9–10 subcultures, primordia was formed on the periphery of nodules (Fig. 5D) and establishing a vascular connection with the nodule (Fig. 5E). Sectional observation through TEM revealed there were lots of mitochondria and extensive rough endoplasmic reticulum (RER) adjacent to dictyosome that were active in producing vesicles (Fig. 5F).

(4) Shoots differentiation: after 11–12 subcultures, early stage of leaf clusters formed with development and elongation of primordia (Fig. 5G), and apical meristems establishing vascular connection with the nodule (Fig. 5H). At this stage, chloroplasts developed a well-organized internal membrane system under TEM observation (Fig. 5I). New elongated shoots containing obvious axillary bud primordia were able to successively develop after transferring to medium containing BA and GA3 (Fig. 6A, B).

The extracellular matrix (ECM)

SEM revealed the presence of a discontinuous amorphous secretions outside the callus surface, and variation in structure, marked as ECM. Compared to ECs covered with numerous compact membranous layers, fibrillar structure and abundant granular mucilage-like secretions (Fig. 7A, B), cells of NECs provided a “peeling” appearance (Fig. 7C). The expansion and multiplication of the surface cells of the nodules caused the membranous layer to burst. As a result, fibrillary structures became visible on the surface of the nodules (Fig. 7D, E). The structure of superficial cells became slightly elongated and regularly arranged leading to the formation of epidermis-like surface at stage of nodular clusters (Fig. 7F), and the ECM structures on the surface gradually decreased and presented fragments appearance (Fig. 7G). However, dense granular mucilage-like secretions formed exclusively on the upper surface of the primordia (Fig. 7H). No ECM structures on the smooth surface of newly formed leaves were detected (Fig. 7I).

Discussion

To our knowledge, this is the first report about histologic and ultrastructural analyses of in vitro regeneration via MNs culture in tree peony. The selection of ECs is prerequisite for in vitro regeneration. In order to distinguish ECs and NECs, morpho-histological and ultrastructural studies were conducted, respectively. There were distinct differences between the two tissues and we identified type I callus as ECs, making it easier to select for subsequent experiment in view of its morphological characteristics, which was basically consistent with previous observations (Moura et al. 2008; Shang et al. 2009; Maadon et al. 2016).
Fig. 4 SEM, histological and cytological observation of callus after 1–6 subcultures on meristematic nodules/shoot induction medium in *Paeonia ostii* ‘Feng Dan’. **A** Callus with small units (black arrow); **B** meristematic cell masses (black arrows); **C** Pre-nodular structures; **D** OCs consisting of a central area of vascularization surrounded by meristematic cell layers; **E** Meristematic nodules; **F** MNs comprised of OCs, a cortical-like area of parenchymatous cells and an epidermal-like area; **G** linear types of organization centers; **H** nested types of organization centers; **I** Cellular ultrastructure of MNs cells; **J** Less starch grains exhibited in the plastids, where contained moderately developed lamellar structures; **PN** pre-nodular structures, **V** vascular system, **OC** organization center, **MN** meristematic nodule, **EP** epidermal-like area, **CP** cortical-like area of parenchymatous cells, **N** nucleus, **NU** nucleolus, **VA** vacuole, **P** plastid, **S** starch grain, **CW** cell wall, **R** rough endoplasmic reticulum, **M** mitochondria, **L** lamella structure.
MNs could be initiated directly from explants in *Cichorium intybus* (Piéron et al. 1993, 1998), *Eucalyptus globulus* (Trindade and Pais 2003), *Populus euphratica* (Ferreira et al. 2009), *Sclerocarya birrea* (Moyo et al. 2009) or indirectly from induced calli, like *Pinus radiata* (Aitken-Christie et al. 1988), *Humulus lupulus* (Batista et al. 2000; Fortes and Pais 2000) and *Acacia mangium* (Xie and Hong 2001). Induction of MNs in tree peony belongs to latter. In addition, callus originated from cell division in both cambial and cortical regions of cotyledon, same as in Qin et al. (2012a) that petiole layers as explant.

Our morpho-histological observation of MNs development in tree peony was similar to descriptions in *Cichorium intybus* (Piéron et al. 1993, 1998), *Humulus lupulus* (Batista et al. 2000; Fortes and Pais 2000), *Populus euphratica* (Ferreira et al. 2009) and *Vriesea reitzii* (Dal Vesco and Guerra 2010). There were three developmental stages leading to the formation of nodules before organogenesis, including pre-nodular structures, MNs and ‘polycenter’ nodules in sequence. Concurrently, MNs have been characterized by distinct internal structures involving a central area of vascularization surrounded by actively dividing meristematic cells.
(OCs), a cortical-like area of parenchymatous cells and an epidermal-like area. In opposition to descriptions in *Populus euphratica* (Ferreira et al. 2009), *Cichorium intybus* (Piéron et al. 1993, 1998) where expansion of ‘polycenter’ nodules in diameter was accompanied by the formation of cracks in the parenchymatous tissue with separation observed along the surfaces of voids, thus multiplying the number of independent nodules, we never observed nodules division. In this report, several nodules were loosely attached to each other and developed into nodular clusters in appearance without detachment. This was consistent with demonstration in *Humulus lupulus* (Batista et al. 2000; Fortes and Pais 2000).

It was reported that shoots originated from epidermis or cortex tissue of nodules in *Humulus lupulus* (Batista et al. 2000; Fortes and Pais 2000), while histological examination revealed that it developed from parenchymal cells around the vascular center in *Cichorium intybus* (Piéron et al. 1993, 1998). Qin et al. (2012b) reported that increased division of the epidermal and subepidermal cells of nodular callus led to shoot-bud regeneration in *P. lemoinei* ‘Golden Era’, however, shoots were proved to regenerate from endogenous parenchyma cells in the vicinity of nodule vascular centers in our research. These distinctions may be related to species differences. Abundant vascular tissues, a predominance feature of nodules, might be closely related to regeneration since the neovascularization following shoots origination was frequent in numerous cases of organogenesis (Piéron et al. 1998; Fortes and Pais 2000; Ferreira et al. 2009). Presence of vascular centers may improve individualization of nodules and can be sign of regeneration.

The histological analysis provided detail evidence for hypothesis that the nodule developmental pathway was distinct to the embryogenic pathway, but highly parallel (McCown et al. 1988). The MNs morphogenetic resulted in the development of monopolar axes, in contrast to the bipolar pattern of somatic embryos differentiation in tree peony (Du et al. 2020).

The most striking ultrastructural feature was the changes of chloroplast morphology and starch content during development under TEM observation. Chloroplasts were degraded to plastids with large amount of starch grains accumulation in ECs cells. Plastids gradually developed into mature chloroplasts as well as starch grain decreased and disappeared step by step. Similar phenomenon had been demonstrated previously in *Bauhinia forficata* (Appezzato-da-Glória and Machado 2004), *Papaver somniferum* (Oveřka et al. 2000) and *Solanum melongena* (Fournier et al. 1995). Starch, one kind of carbohydrate, considered to be the primary source of energy for cellular proliferation and growth, thus being consumed during in vitro morphogenesis (Moura et al. 2008). In addition, the storage of starch, abundant in ECs and shortage in NECs, probably indicated the acquisition of embryogenic potential (Pinto et al. 2010; Ribas et al. 2011), and they might function as signal molecules in signal transduction and gene regulation (Luis and Scherwinski-Pereira 2014).

There was also a remarkable observation during differentiation of secretory dictyosome located around abundant extensive RER, surrounded with plentiful complete or dividing mitochondria. This phenomenon had been intensively reported (Aitken-Christie et al. 1988; Appezzato-da-Glória and Machado 2004; Diego et al. 2012). The active operation of inner membrane systems may reveal a high synthesis capacity associated with in vitro morphogenesis.

SEM observation in this report revealed the presence of ECM, which consisted of membranous, fibrillar and granular structures. Similar structure had also been reported in *Centella asiatica* (Lai et al. 2011), *Actinidia delicosa* (Popielarska-Koniczna et al. 2006, 2010) and *Triticum*...
aestivum (Konieczny et al. 2005). The chemical compositions and functions of ECM are still uncertain. It might be pectin polymers (Verdeil et al. 2001; Konieczny et al. 2007), arabinogalactan proteins (Konieczny et al. 2007) and lipid (Popielarska-Konieczna et al. 2008). In addition, the formation of ECM might be a stress response to in vitro conditions or resulted from unsuitable pre-treatment (Konieczny et al. 2005). On the other hand, ECs could be identified with the ECM since it was suggested to be an indicator of cells potential for regeneration (Namasivayam et al. 2006; Popielarska-Konieczna et al. 2006; Lai et al. 2011; Yusoff et al. 2012). However, ECM was observed both on the ECs and NECs in Oryza sativa (Bevitori et al. 2014) and Helianthus tuberosus (Pilarska et al. 2014) regardless of its morphogenetic competence. This discrepancy may result from genotype differences. In our study, three kinds of ECM structures were abundant on ECs, but absent from NECs. Furthermore, dense granular secretions were exposed on the surface of the primordia exclusively, which was aligned with result of some thesis that the appearance of ECM covering the surface was linked to the induction of morphogenesis (Konieczny et al. 2005) and can serve as a structural marker of somatic embryogenesis (Namasivayam 2007) or organogenesis (Popielarska-Konieczna et al. 2006). The ECM was also considered to play a vital role in regulation of signal, movement of nutrients, protection of surface structure during plant development and morphogenesis (Popielarska-Konieczna et al. 2010).

The earliest symptom of differentiation was the distinctive presence of nodular clusters with elongated and regularly

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**Fig. 7** SEM observations of the extracellular matrix (ECM) structures during meristematic nodules induction and shoot organogenesis in *Paeonia ostii* ‘Feng Dan’. A Membranous layer (black asterisks) and fibrillar structures (black arrow); B Granular mucilage-like secretions (black arrows) on embryogenic callus surface; C Cells on surface of non-embryogenic callus provided a ‘peeling’ appearance; D, E ECM structures on MNs show transition from membranous layers to fibrillar structures (black arrows); F Epidermis-like surface of nodular clusters; G ECM structures gradually decreased with fragments appearing on nodular cluster; H Dense granular structures on the primordium region; I Smooth leaf surface cells showing the absence of ECM structures.
arranged superficial cells under SEM observation, leading to the formation of epidermis-like surface, where primordia initiated soon after. This corresponded with findings in *Oryza sativa* (Bevitori et al. 2014) and *Actinidia deliciosa* (Popielarska-Konieczna et al. 2011). The noticeable structure might be closely related to the origin of primordia and could be serviced as an indicator of differentiation (Brisibe et al. 1992).

**Conclusion**

This is the first report covering histological and cytological analyses involved in MNs morphogenesis system in *P. ostii* ‘Feng Dan’. The histological study revealed a developmental sequence leading to the formation of MNs and shoots regeneration, including callus induction, pre-nodular structures, MNs, nodular clusters, shoots differentiation. In addition, the knowledge obtained by SEM and TEM about the developmental states of MNs morphogenesis can be useful for additional optimization of the in vitro regeneration protocol and form the basis for further molecular analysis or genetic transformation in tree peony.

**Author contributions** LX conducted the experiments and written the manuscript. FYC and YZ revised the manuscript. All authors read and approved the final manuscript.

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**Declarations**

**Conflict of interest** The authors declare that they have no conflict of interest.

**Research involving human and/or animal participants** This article does not contain any studies with human participants or animals performed by any of the authors.

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