Paeonia ostii ‘Feng Dan’ plant regeneration through direct organogenesis and direct meristematic nodule culture

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Abstract

Tissue culture is preferred for solving the shortcoming of low efficiency in terms of conventional propagation ways in tree peony, an economically important woody plant in China with various purposes. However, callus differentiation is hard to obtain during in vitro regeneration. Meristematic nodule (MN) is a favorable way capable of overcoming this problem, but possesses a lengthy process. Direct organogenesis excluding the callus step is needed to simplify the procedure. This study firstly presented a protocol of direct organogenesis and direct MNs induction and differentiation using cotyledon explant for in vitro regeneration of P. ostii ‘Feng Dan’. The highest direct MNs induction rate (41.67%) and frequency of direct organogenesis (DO) (66.67%) was achieved under the following procedure. The explants were pretreated in dedifferentiation induction medium (DIM) [Murashige and Skoog (MS) medium with 2.27 µM thidiazuron (TDZ)+5.37 µM α-naphthylacetic acid (NAA)] for 10 days, and then the cotyledons without callus induced were transferred to differentiation medium (DM) [Woody plant medium (WPM) containing 2.02 µM N-(2-chloro-4-pyridyl)-N-phenylurea (CPPU)+2.27 µM TDZ and 4.04 µM CPPU+4.54 µM TDZ] respectively, with 6 subcultures, 90 days in total. The regenerated shoots rooted and transplanted successfully. Histological study confirmed the process of DO and direct MNs induction, and revealed that shoots and MNs were originated from increased division of meristematic cell under cortical tissue, as well as from actively divided meristematic cells around vascular center. Moreover, shoots regenerated through MNs differentiation were originated from the epidermal and subepidermal cells. This study is an innovation and supplement in the field of in vitro regeneration in tree peony, and will be conductive to clonal micropropagation, fundamental studies of developmental biology and genetic transformation.

Introduction

Tree peony (Paeonia sect. Moutan) is valued as an ornamental plant rich in colors and patterns, also used as medicinal and oil production plant (Yu et al. 2016). However, mass propagation of this species is limited by the characteristics of its conventional propagation methods, low efficiency and long period. Tissue culture is a useful way to solve this problem.

The research of tree peony concerned with tissue culture date back to 1984 (Li et al. 1984), and over the past four decades, it has been widely recognized that difficulty in differentiation of callus is a key issue that hinders in vitro regeneration (Du et al. 2020b; Wen et al. 2020). For example, several studies on somatic embryogenesis of tree peony have been reported, but no available regeneration system published because of rare differentiation from callus in indirect pathway (Zhu et al. 2018; Du et al. 2020b), so did organogenesis from callus (Wen et al. 2020). Breakthrough, an available regeneration system of tree peony via meristematic nodules (MN) culture, derived from callus, has been described previously (Xu et al. 2022a). MN shown resemblance in appearance with somatic embryo, but could be distinguished from histological aspect (Xu et al. 2022b; Haensch 2004). It possessed high potential in micropropagation, genetic transformation, and bioreactor for producing chemical components (McCown et al. 1988; Batista et al. 2008). Nevertheless, the system yielded the disadvantage of complex procedure and long period as
a result from the cumbersome process of callus and MNs induction. Hence, the regeneration system via MNs culture not involving a strict callus phase is needed to streamline the lengthy process. Similar protocols have already been announced in some literature (Piéron et al. 1993, 1998; Trindade and Pais 2003; Moyo et al. 2009).

Direct organogenesis (DO) originated from explant without callus period have been declared in some plants, such as *Anaphalis hancockii* (Geng et al. 2023), *Solanum melongena* (García-Fortea et al. 2020), *Hypericum perforatum* (Ravindran et al. 2023). This system is relatively plainer and possesses a shorter cycle. Whereas there were scarce reports carried out about this protocol in tree peony. Overall, innovative breakthroughs concerning in vitro shoot organogenesis excluding the callus step are needed to simplify the procedure.

The present work aimed to achieve shoot organogenesis with two novel pathways avoiding callus step, shoots emerged directly from explants or explants-derived MNs. Histological analyses were undertaken to reveal the characteristics of develop stages in morphogenetic process. This study could be a significant innovation and supplement in the field of in vitro regeneration in tree peony.

**Materials and methods**

**Plant material**

In August 2022, seeds at 90 days after anthesis from five-year-old *P. ostii ‘Feng Dan’* plants were obtained from Beijing Guose Peony Garden in Beijing, China (40°45′N, 115°97′E). The collected seeds were placed in the refrigerator (−4°C) for at least 2 months.

**Explant sources**

After washing under running tap water for 10 mins, the seeds were soaked in commercial liquid detergents (1% v/v; 5 min). Afterwards, seeds with clean surface were sterilized in clean bench by dipping in ethanol (70% v/v; 1 min) first, and then NaOCl (2% v/v; 15 min). Finally, the seeds were rinsed with sterile distilled water for at least three times before inoculation. According to the method adapted from Xu et al. (2022a), zygotic embryos were inoculated on MS medium containing 2.57 µM 6-benzyladenine (BA) and 2.89 µM gibberellin (GA₃) for germination, the cotyledons after 15 days of culture were used as explants.

**Medium and culture conditions**

All the cultures were grown on Woody plant medium (WPM), Murashige and Skoog medium (MS) and 1/2 MS (half-strength macroelements). The basal media containing 3% sucrose and 0.7% agar (Biosharp, Beijing, China). The pH of the media was pre-adjusted to 5.8-6.0 prior to sterilisation (at 120°C and 115 kPa for 20 min). All cultures were kept in a growth room at temperature of 23 ± 2°C under a 16/8 h photoperiod using cool white light (25 µmol·m⁻²·s⁻¹).
Direct organogenesis (DO) and direct meristematic nodule (MN) induction

To study the effects of culture time and plant growth regulators (PGRs) in dedifferentiation induction medium (DIM) on the DO and direct MN induction, the explants were cut into pieces (1×1 cm) and inoculated on DIM [MS + 2.57 µM BA + 5.37 µM α-naphthylacetic acid (NAA)] for different days (0, 5, 10 and 15 days) firstly. The excised cotyledon explants were placed with their abaxial face down in contact with the medium, and kept in dark conditions. Secondly, DIM containing different PGR combinations [2.57 µM BA + 5.37 µM NAA, 2.02 µM N-(2-chloro-4-pyridyl)-N-phenylurea (CPPU) + 5.37 µM NAA, 2.27 µM thidiazuron (TDZ) + 5.37 µM NAA] was used. The excised cotyledon explants were cultured for 10 days respectively. Afterwards, the cotyledons were transferred from DIM to differentiation medium (DM) [WPM + 2.27 µM TDZ], with subculture times of 15 days. The frequency of DO (%) and direct MN induction rate (%) were evaluated 6 subcultures after transfer. Each treatment consisted of 16 explants, and the experiment was repeated three times. The frequency of DO is expressed as the average percentage of explants/cotyledons that differentiated shoots directly over total number of explants/cotyledons. The direct MN induction rate is presented as the mean number of explants/cotyledons induced MNs directly over total number of explants/cotyledons.

The cotyledons from best DIM after cultured for optimal culture time obtained above were subculture in DM. To screen optimal PGRs in DM for DO and direct MN induction, three cytokinins [2.57 µM BA, 2.02 µM CPPU and 2.27 µM TDZ] were employed respectively. After preliminary screening of suitable hormones, an orthogonal test involving two factors (CPPU and TDZ) and three levels of concentration (1.00, 2.02, 4.04 µM; 1.14, 2.27, 4.54 µM) was undertaken.

Shoot elongation, rooting and acclimatization

The nodules induced directly from explants were transferred to medium [WPM + 2.02 µM CPPU + 2.27 µM TDZ] (Xu et al. 2022a) for leaf clusters differentiation with 3 subcultures, 30 days in total. The cotyledons with leaf clusters (DO), as well as nodules with leaf clusters (direct MN induction and differentiation) were placed in shoot elongation medium [WPM + 1.29 µM BA + 0.58 µM GA₃] respectively with 2 subcultures, 60 days in total.

For in vitro root formation, micro-shoots about 1–3 cm in length were transferred to 1/2 MS basal medium supplemented with 4.92 µM indole-3-butyric acid (IBA) and 11.34 µM putrescine (Wang et al. 2016). Micro-shoots on root induction medium were cultured in the dark for the first 8 days at 4°C and then 30 days at 24 ± 1°C, prior to being cultured in 1/2 MS basal medium supplemented with 0.4% activated carbon for 20 days under a 16/8 h photoperiod using cool white light (25 µmol·m⁻²·s⁻¹). Plantlets with well-developed shoots and roots were then removed from the agar medium and potted in plastic pots containing autoclaved substrate (vermiculite, peat, and perlite in a 1:1:1 volumetric ratio). Agar was removed from the roots thorough carefully washed with running water prior to transplanting.
The pots were placed in a culture chamber at 20 ± 1°C under a 16/8 h photoperiod using cool white light (25 µmol·m⁻²·s⁻¹).

**Histological analysis**

To study the development stage of direct organogenesis and direct MN induction and differentiation, fresh samples [cotyledons cultured in DIM for 15 days, 1×1 cm; cotyledons cultured in DM for different times of subcultures (1, 2, 3, 4, 5 and 6 times), cotyledons with nodules, cotyledons with leaf clusters, nodules with leaf clusters, 1×1×1 cm] were fixed for 48 h in the FAA solution (50% alcohol, glacial acetic acid, and formaldehyde at a ratio of 18:1:1). The permanent preparation was made based on the method adapted from Xu et al. (2022b). Sections 8–10 µm in thickness were obtained using a rotary microtome, and stained with fast green (0.1%) and safranin (0.1%). The prepared slides were studied with Leica model DM500 microscope.

**Statistical analysis**

The data were subjected to analysis of variance (ANOVA) following Duncan's multiple range test to detect significant differences (p ≤ 0.05) in the mean using SPSS 23.0 (SPSS Inc., Chicago, USA), after transforming the percentage values using arcsine transformation. Variability in the data was expressed as the mean ± standard deviation.

**Results**

**Morphological and histological study on DO from cotyledons**

The detailed morpho-histological characterization of explants during in vitro direct organogenesis was conducted for the first time in tree peony to determine the timing and tissue origin of the regenerants. Compared to the original state, active-divided subepidermal cells and meristematic cells around vascular centers were observed in cotyledon after pretreated in DIM (Fig. 1A). Incubation in DM for 1-2 times resulted in obviously swelled and elongated of cotyledon (Fig. 1B). In particular, the occurrence of DO was observed at two positions. Strip protuberances gradually apparent on the surface of cotyledons unevenly (Fig. 1C), and visible swell formed at the cotyledon petiolar cut edge (Fig. 1D).

Sections revealed subepidermal cells vigorously proliferated with large and clear nuclei leading to the formation of strip protuberances (Fig. 1E). Meanwhile, transverse sections of swell showed rapid division of meristematic cell under cortical tissue and around vascular bundles filling up the expansive intercellular spaces (Fig. 1F). The volume of protuberances in both positions was increased irregularly after 3-4 times of incubation, and primordia were shown up. Direct connections between vascular tissue inside primordia and explants were detected in strip protuberances (Fig. 2A, B, C). However, histological observation of swell demonstrated that primordia was initiated inside and developed towards the surface of cotyledons (Fig. 2D, E). Leaf clusters occurred at two positions after 5-6 subcultures, protuberances on the surface of cotyledon (Fig. 3A, B) and swell on the edge (Fig. 3C).
Subsequently, apical meristems could be found out (Fig. 3D). Shoots developed from leaf clusters successively after transferring to shoot elongation medium (Fig. 3E).

**Morphological and histological study on direct MNs induction and differentiation**

Histological examination confirmed direct MNs induction. The process of direct MNs induction and DO was occurred simultaneously. Rapid cell division were initiated in the adaxial portion of subepidermal cells, leading to small globular protuberances arose from the surface of cotyledons within 1-2 times of incubation in DM (Fig. 4A, B). The protuberances significantly expanded during 3-4 subculture (Fig. 4C, D), accompanied with massively formation of vascular tissue (Fig. 4E). Under histological observation, the large protuberances gradually developed into MNs, composed of cortical, epidermal layer cells, and various organization centers, like nested and linear (Fig. 4F, G). During 5-6 times of incubation, nodules proliferation occurred with a special way like budding. Therefore, nodular clusters were appeared since different sizes of nodules were gathered (Fig. 5A). Owing to vigorous division of the epidermal and subepidermal cells, primordia emerged from nodules (Fig. 5B, C, D). When nodular clusters were transferred to leaf cluster differentiation medium, leaf clusters gradually observed on the surface (Fig. 5E). Similarly, shoots arose from leaf clusters in succession after incubated in shoot elongation medium (Fig. 5F).

The eligible shoots induced both from DO pathway and direct MN culture developed roots after placement on the rooting medium (Fig. 5G), and the rooted plantlets were acclimatized successfully (Fig. 5H).

**Effect of culture time in DIM on DO and direct MNs induction**

DO or direct MN induction were failed to be observed from the cotyledons not cultured in DIM for dedifferentiation (Fig. 6). Pre-culture in DIM positively affected the formation of DO and direct MNs induction. the frequency of DO and the direct MN induction rate were gradually increased with dedifferentiation time, and the highest frequency of DO (43.75%) was achieved when cotyledons explants were pretreated in DIM for 10 days, but with no significant difference between 10-15 days of treatment. The direct MN induction rate of all treatments was generally low, but treatments of 10-15 days were higher than that of 0-5 days.

**Effect of PGRs in DIM on DO and direct MNs induction**

As shown in figure 7, the frequency of DO from cotyledons pre-cultured in DIM supplemented with 2.02 µM CPPU+5.37 µM NAA (50.00%) and 2.27 µM TDZ+5.37 µM NAA (52.08%) was significantly higher that of 2.57 µM BA+5.37 µM NAA (39.58%). Combination of TDZ and NAA was the treatment that showed the highest frequency of DO. However, there was no significant difference on the direct MNs induction rate among treatments (10%).

**Effect of PGRs in DM on DO and direct MNs induction**
In the preliminary experiment, DO and direct MNs induction only occurred in DM containing TDZ or CPPU, but not in presence of BA (Fig. 8). The results indicated that the frequency of DO in treatment with CPPU (41.67%) and TDZ (45.83%) was conspicuously higher than BA (0%), but present no significant difference between two of them. Meanwhile, the direct MN induction rate of treatment with CPPU (10.42%) superior to TDZ (4.17%).

In subsequent steps, the best performance of direct MN induction rate (41.67%) was obtained at 2.02 µM CPPU and 2.27 µM TDZ, while the optimal concentration of CPPU and TDZ was 4.04 µM and 4.54 µM respectively in terms of the frequency of DO (66.67%) (Table 1). Variance analysis demonstrated that there was a significant response for CPPU and TDZ concentration on the frequency of DO ($p < 0.01$), as well as their interaction (Table 2). In terms of the direct MN induction rate, CPPU and its combination with TDZ remained significant effect, but TDZ alone not influenced ($p > 0.05$).

The number of regenerated shoots originated from both DO and direct MNs culture pathway varying from three to seven per explant. The rooting rate remains around 50%, and the survival rate was kept between 30-40%.

**Discussion**

This is the first report of DO from cotyledon explants without callus period in tree peony. In this experiment, leaf cluster initiation was observed at cut site of the proximal end of cotyledon, as well as the paraxial surface, which in agreement with description in some literature (Huang et al. 2014; Debnath et al. 2018). Simultaneously, the occurrence of direct organogenesis was confirmed by the histologic analysis that shoots originated from increased division of meristematic cell under cortical tissue, as well as meristematic cells around vascular centers inside of swell on the edge of the cotyledon. Similar phenomenon was revealed previously, but shoot initiation was varied. For instance, from the upper epidermal cells and their inside parenchyma cells in *Capsicum annuum* (Gao et al. 2021), from the epidermal tissue in *Neolamarckia cadamba* (Huang et al. 2014), from the epidermal and subepidermal cells in *Cucumis melo* (Cai et al. 2002), which was called for exogenous initiation. Besides, buds could regenerate from endogenous meristematic cells around vascular centers (Sarkar and Jha 2017). Thus, the origin of shoots through the DO pathway is endogenous and exogenous coexist in tree peony.

On the other hand, this is also the first report of direct MNs induction and differentiation without callus phase in tree peony. Histological examination confirmed this morphogenic response. This is contrary to previous conclusion that callus formation was necessary for MNs induction in tree peony (Xu et al. 2022a). In fact, there were two morphogenesis pathways has been reported on MNs induction, direct (Moyo et al. 2009; Ferreira et al. 2009; Piéron et al. 1998) and indirect with callus formation (Fortes and Pais 2000; Batista et al. 2000). Therefore, two pathways were existed side by side in tree peony.

In this model of direct MNs culture on tree peony, cells competent for nodule induction was located mainly in meristematic cell under cortical tissue with histological observation, and new formed vascular system developed inside nodules subsequently, which was basically consistent with results in some...
reports (Moyo et al. 2009; Ferreira et al. 2009). However, there were different conclusion in *Cichorium intybus* that the cambium of nodule was originated from the procambium of leaf, and the parenchyma and periderm cork cell layers were originated from the fascicular parenchyma and bundle sheath tissues of leaf in direct (Piéron et al. 1998). These distinctions might be related to species differences. Additionally, it was documented that shoots originated from epidermis or cortex tissue of nodules in *Humulus lupulus* (Batista et al. 2000; Fortes and Pais 2000), or from parenchymal cells around the vascular center in *Cichorium intybus* (Piéron et al. 1993, 1998). In contrast to previous text that shoots were regenerated from endogenous parenchyma cells around nodule vascular (Xu et al. 2022b), our histological examination revealed that increased division of the epidermal and subepidermal cells of nodules led to shoot regeneration. Similar observation has been recognized by Qin et al. (2012) in *P. lemoinei* ‘Golden Era’. This difference might attribute to insufficient number of sections.

In our studies, DO and direct MNs induction took place synchronously, and highly parallel at the early stage. Both induced protuberances initiated from subepidermal cells, but shared different shapes. When it comes to DO pathway, strip protuberances became apparent on the upper part surface of cotyledons, and the appearance of swell growth with irregular shape was observed on the edge. On the contrary, the nodules exhibited a global-shaped structure at the beginning of their development, similar to globular somatic embryos. The histological analysis could provide detail evidence for distinguishing (Xu et al. 2022b; Haensch 2004). Subsequently, the volume of swell at the cut site of cotyledon gradually increased, so did the nodules. Nevertheless, they were remarkable distinct in terms of appearance (Fig. 2D; Fig. 4D) and location. The swell located just at the cut end of cotyledon explants, same as *Garcinia mangostana* (Qosim et al. 2015), but nodules were not restricted. In addition, there were more plenty of autogenetic vascular tissues inside the nodules compared to swell (Fig. 2E; Fig. 4E).

Pretreatment in DIM (dedifferentiation) before callus formation played a dominant role in this protocol. With the enhancement of dedifferentiation time (0–15 days), the differentiation rate in DM improved. It was speculated that cells with high activity after losing their original characteristic structure and function within dedifferentiation time, could trigger a particular developmental fate when recognized a single inductive signal. Specifically, dedifferentiated subepidermal cells and meristematic cells around vascular centers could developed into primordia or nodules after transferring cotyledons from DIM to DM supplement with highly active cytokinin. The results emphasized the importance of dedifferentiation step of explants in DIM enriched with auxin. It is well known that auxin is essential for apical meristem formation. In detail, auxins and downstream transcriptional regulation interfere with the structural elements of the cell wall to induce specific morphogenetic events, the absence of auxins in the pretreatment inhibited the morphogenic process (Traas 2019). Furthermore, cross-talk with other signaling pathways, cytokinin in particular, is crucial in organ regeneration (Vernoux et al. 2011; Huang et al. 2014). Similarity, the procedure was developed in *Eucalyptus nitens* (Ayala et al. 2019) and in *Prunus cerasifera* (Carmona-Martin and Petri 2020), that pre-treatment with auxins under dark condition and subsequent transfer to medium rich in cytokinin in light conditions, and considered to be appropriate in recalcitrant specie for direct regeneration.
Effect of PGRs in DM on direct regeneration was dependent on PGRs type and concentration (Bao et al. 2017; Huang et al. 2014), genotype (García-Forte et al. 2020) and explant type (Geng et al. 2023). According to Xu et al (2022a), CPPU and TDZ, two kinds of cytokinin with high activity, played remarkably positive roles in MNs induction and shoots regeneration from callus. Meanwhile, DO and direct MNs induction only occurred in DM containing TDZ or CPPU in this research, but not in presence of BA, which further supported previous speculation that high level of cytokinin was indispensable for differentiation (Xu et al. 2022a; Meng et al. 2017). The result that TDZ + NAA, CPPU + NAA treatment in DIM had a better effect on the induction of MNs and DO than BA + NAA treatment also consistent with this conclusion. In contrast, the cytokinin suitable for direct regeneration was varied among other plant species, like BA for *Garcinia mangostana* (Qosim et al. 2015), ZR for *Solanum melongena* (García-Fortea et al. 2020), TDZ for *Rhododendron* (Hebert et al. 2010), and combination of KT and BA for *Anaphalis hancockii* (Geng et al. 2023). Besides, mixed use of CPPU and TDZ was more effective than single one in direct regeneration, which was in coincidence with other result (Gao et al. 2021).

The developmental direction of differentiation was varied with cytokinin type and concentration. For example, the epidermal cells of leaves developed into nodular callus if stimulated by combination TDZ and BA, while shoot originated with BA stimulation alone. What's more, the optimum treatment for direct shoots induction was achieved at a concentration of 22.2 µM BA, but the use of BA (> 44.4 µM) inhibited the formation and elongation of shoots (Qosim et al. 2013, 2015). In the present study, both of CPPU and TDZ concentration had significant effect on the frequency of DO (p<0.01), but TDZ showed no significant effect on the direct MN induction rate (p>0.05). Similar result has been concluded in tree peony that CPPU was considerably effective in promoting MNs induction from callus (Xu et al. 2022a), and in herbaceous peony that TDZ was appropriate for direct organogenesis from cotyledon explants (Zhao et al. 2017). Concurrently, the best performance of DO and direct MNs induction was presented on CPPU and TDZ combination at different level of concentration in our study, and higher level suitable for DO. Thus, researchers can make different choices in concentration formulas based on varied purposes.

The protocol achieved in this essay can greatly shorten the period needed for regeneration by skipping the callus stage. More specifically, it takes approximately 6 months to obtain differentiated shoots in DO pathway, and this regeneration system through direct MNs induction from explants and differentiation can save approximately 2 months compared to previous cycle (Xu et al. 2022a). Moreover, MNs of tree peony in vitro work as storage organs like corms or tubers, and scale-up multiplication of MNs can be reached in liquid medium (Zhong et al. 2011). Therefore, optimization of in vitro regeneration can be achieved with procedure that MNs induced from explant directly multiplicatied in liquid environment, and then differentiated in solid medium. This hypothesis has been realized in *Ananas comosus* var. *comosus* (Scherer et al. 2013), *Vriesea reitzii* (Dal Vesco and Guerra 2010), and *Charybdis numidica* (Kongbangkerd and Wawrosch 2003). We have obtained preliminary evidence that nodules proliferated in liquid medium could finish differentiate in solid medium, but further optimization of culture conditions still underway.

**Conclusion**
This study firstly presented a protocol of in vitro regeneration through DO and direct MNs culture in *P. ostii* ‘Feng Dan’. This protocol includes two pathways at the same time. The explants need pretreated in DIM in both ways, and then occurred simultaneously in DM. Shoots regenerated directly from explants (DO pathway) and from explants-derived MNs could rooted, and the rooted plantlets were acclimatized successfully. In addition, histological study revealed the developmental sequence and tissue origin of the regenerants. This protocol simplified the differentiation process and will be beneficial to the clonal micropropagation, fundamental studies of developmental biology and genetic improvement of tree peony.

**Abbreviations**

BA, 6-benzyladenine; CPPU, N-(2-chloro-4-pyridyl)-N-phenylurea; DM, Differentiation medium; DIM, dedifferentiation induction medium; DO, Direct organogenesis; GA₃, Gibberellin; IBA, Indole-3-butyric acid; MN, Meristematic nodule; MS, Murashige and Skoog; WPM, Woody plant medium; NAA, α-naphthylacetic acid; PGR, Plant growth regulator; TDZ, Thidiazuron;

**Declarations**

**Compliance with ethical standards**

**Funding** The study was supported by the National Natural Science Foundation of China (32302598), and Natural Science Foundation of Hubei Province of China (2023AFB509).

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

**Ethical approval** This article does not contain any studies with human participants or animals performed by any of the authors.

**Data availability** The data supporting the findings of this study are available with Li Xu and can be made available upon request.

**Author contribution** CCF and KXL conducted the experiments and written the manuscript. LX designed the experiment and revised the manuscript. ZJD, SMD, JTL and JLM assisted in guiding the experiments. All authors read and approved the final manuscript.

**References**


### Tables

Table 1 Screening of CPPU and TDZ concentration for the frequency of DO and the direct MN induction rate

<table>
<thead>
<tr>
<th>CPPU (µM)</th>
<th>TDZ (µM)</th>
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<th>The direct MN induction rate (%)</th>
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Different letters within a column show significant differences by Duncan's multiple range tests (p ≤ 0.05). Each data represent mean ± standard error.
Table 2 Variance analysis of CPPU and TDZ concentration on the frequency of DO and the direct MN induction rate

<table>
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<th>Source of variance</th>
<th>The frequency of DO (%)</th>
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Figures
Figure 1

Morphological and histological observation of direct organogenesis (Early stage)

A. Active-divided subepidermal cells (black arrow) and meristematic cells around vascular centers (hollow black arrows) were observed in cotyledon after pretreated in DIM; B. Cotyledons obviously swelled and elongated after cultured in DM for 1-2 times; C. Strip protuberances were observed on the surface of
cotyledons unevenly (black arrow); D. Visible swell formed at the cotyledon petiolar cut edge (black arrow); E. Subepidermal cells proliferated extensively to form protuberances (black arrow); F. Rapid division of meristematic cell under cortical tissue (black arrow) and around vascular bundles (hollow black arrows) filling up the intercellular spaces of swell. Abbreviations: V. Vascular bundle; EP. Epidermis.

Figure 2
Morphological and histological observation of direct organogenesis (Middle stage)

A-B. After 3-4 times of culture in DM, the volume of protuberances was increased, and leaf primordia were appeared on the surface of cotyledon (black arrow), and direct connections between vascular tissue inside primordia and explants were detected (hollow black arrows); C. Enlarged details of vascular tissue in Figure A (hollow black arrows); D-E. After 3-4 times of culture in DM, the volume of swell was increased irregularly, and bud primordia were observed inside, and developed towards the surface of cotyledon. Abbreviations: V. Vascular bundle; LP. leaf primordia; P. bud primordia.
Figure 3

Morphological and histological observation of direct organogenesis (Later stage)

A-B. Early stage of leaf clusters observed from the surface of cotyledon after 5-6 subcultures in DM; C. Leaf clusters occurred from the swell at the edge of cotyledon; D. Apical meristems (black arrow) can be
detected; E. Shoots developed from leaf clusters successively after transferring to shoot elongation medium.

Figure 4

**Morphological and histological observation of direct MNs induction**

A. Small globular protuberance (black arrow) arose from the surface of cotyledons within 1-2 times of incubation in DM; B. Rapid cell division were initiated in the abaxial portion of subepidermal cells leading to globular protuberance (black arrow); C-D. The protuberances (black arrow) significantly expanded after 3-4 subculture; E. MN composing of cortical, epidermal layer cells, and various organization centers; F.
Nested types of organization center; G. Linear types of organization center. Abbreviations: V. Vascular bundle; EP. epidermal layer cells; CP. cortical cells; OC. organization centers.

Figure 5

Morphological and histological observation of nodular cluster differentiation

A. Explants with nodular cluster induced directly after 5-6 subculture in DM; B. Primordia (black arrow) occurred on the surface of nodule; C. Nodular cluster with primordia initiated (black arrow); D. Bud
primordia (black arrow); E. Leaf clusters were developed from nodule; F. Shoots developed from leaf clusters successively after transferring to shoot elongation medium.; G. Shoots developed root; H. The rooted plantlets were acclimatized successfully. Abbreviations: EP. epidermal layer cells; CP. cortical cells; OC. organization centers.

Figure 6

Legend not included with this version
Figure 7

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Figure 8

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